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Biological Pretreatment of Mexican Caribbean Macroalgae Consortia Using Bm-2 Strain (*Trametes hirsuta*) and Its Enzymatic Broth to Improve Biomethane Potential

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Abstract: The macroalgae consortium biomass in the Mexican Caribbean represents an emerging and promising biofuel feedstock. Its biological pretreatment and potential for energetic conversion to biomethane were investigated, since some macroalgae have hard cell walls that present an obstacle to efficient methane production when those substrates are used. It has been revealed by anaerobic digestion assays that pretreatment with a Bm-2 strain (*Trametes hirsuta*) isolated from decaying wood in Yucatan, Mexico was 104 L CH₄·kg VS⁻¹; In fact, the fungal pretreatment produced a 20% increase in methane yield, with important amounts of alkali metals Ca, K, Mg, Na of 78 g/L, ash 35.5% and lignin 15.6%. It is unlikely that high concentrations of ash and alkali metals will produce an ideal feedstock for combustion or pyrolysis, but they can be recommended for a biological process.

Keywords: biological pretreatment; macroalgae consortium; biomethane potential; *Trametes hirsuta*

1. Introduction

Although there are a large number of species of macroalgae, the majority of shoreline inundation incidents are caused by two genera: *Ulva* is a green macroalga, which causes green tides, and *Sargassum*, a golden/brown floating or pelagic macroalga responsible for golden tides, especially in the Caribbean and West Africa [1].

The genus *Sargassum* contains over 350 species. Two of these, *S. natans* and *S. fluitans*, are holopelagic and the major contributory species in golden tides. Both of these have a vegetative reproduction process, and do not physically connect to the ocean floor during their lifecycle [1].

Pelagic *Sargassum* (gulfweed) has been called a floating jungle or floating golden rainforest, as it provides a habitat for a wide range of invertebrates, fishes, sea turtles, birds, and mammals: over 100 species of fish and 145 species of invertebrate have been found linked to it. *Sargassum*'s importance lies not only in its ecological role, but also in the part it plays worldwide in oceanic carbon sequestration: the *Sargassum* of the Sargasso Sea is a net sink of CO₂, and represents 7% of the planet's net "carbon pump". It is also the largest accumulation of macroalgae in the world, containing a total biomass of 10 million tons. Approximately one million tons of *Sargassum* passes through the Florida Straits from the Gulf of Mexico towards that sector of the Atlantic Ocean known as the Sargasso Sea each year [2].

During the 2015 Caribbean inundation, 10,000 wet tons of macroalgae were being cast up onto Caribbean island beaches every day. The precise cause of the *Sargassum* inundations in the last few years is not completely understood, although it is believed that both climate change and coastal sea eutrophication are involved. Nor is it clear whether the large golden tides of 2011–2015 will constitute a phenomenon that will be repeated in following years [1].

Sargassum inundations have a part to play in maintaining the stability of beaches, and they also provide a food source for beach and dune vegetation. *Sargassum* cast up on a beach is not thought to present any risk to human health, although beach users may dislike the presence of large quantities. It can also have unwelcome environmental effects, suffocating animals such as turtle hatchlings. Tourism provided more than 80% of the Caribbean region's GDP in 2014, bringing \$29.2 billion in local spending. On the other hand, removing *Sargassum* from beaches or stopping its arrival might prove extremely expensive. Taking as a basis the figure of \$5 million spent on cleaning Mexican beaches, it is possible to calculate that at least \$120 million would be required to remove *Sargassum* inundations throughout the Caribbean. Clean, renewable biomass from the ocean might provide an outstanding addition to our present range of alternative energy sources [3,4]. At the present time, the application and viability of anaerobic digestion technology on macroalgae in the long term have not been established, and it is worth researching both mono- and codigestion methods. The currently available literature on monodigestion of seaweeds in continuous processes is limited [5]. Specifically, it is possible to convert macroalgae into biogas (60% methane) through anaerobic digestion. Algal biogas could potentially reduce greenhouse gas emissions by more than half and fossil depletion by nearly 70%, in comparison with natural gas [6].

White rot fungi have a well-known ability to decompose a wide spectrum of environmentally persistent xenobiotics and organopollutants. Similarly, recent tests have been made on biological methods such as using enzymes. They are considered relatively cheap, environmentally friendly pretreatments for improving the anaerobic biodegradability of macroalgal and microalgal biomass. Choice of enzymes is determined by the main compounds forming the macroalgal and microalgal cell wall—specifically, cellulose, hemicelluloses, pectin, glycoproteins, and even lignin. Depending on the culture conditions, Ligninolytic fungi generate nonspecific intracellular and extracellular enzymes. The presence of various inducers leads to the production of laccases, and how the latter affect metabolic activity and cell growth depends on environmental conditions and specific regulatory mechanisms. The white-rot fungus *Trametes* sp. is one of the best-known laccase-producing fungi [7,8].

Thus, the objective of this work was to evaluate the potential of biogas production from macroalgal biomass of the Mexican Caribbean in tests of biochemical methane potential (BMP), using a strain Bm-2 (*Trametes hirsuta*) isolated in Yucatan, Mexico, and its enzymatic broth as biological pretreatments, as well as structural changes occurred during the pretreatment of this biomass.

2. Materials and Methods

2.1. Macroalgae Consortium Biomass and Characterization

Samples of a mixture of macroalgae biomass was manually collected on the shore in Progreso (Yucatan, Mexico) in January 2017, in order to reproduce the conditions of harvesting readily available biomass on the beach. The samples were washed with tap water several times to remove impurities like salts and sands, and then dried at 105 °C (APHA 2005). The biomass was stored in a cold room. The characteristics of macroalgae consortiums are summarized in Table 1.

Table 1. Main characteristics of macroalgae biomass.

Component Name	Average (%)
Lignin	15.6 ± 0.51
Cellulose	31.2 ± 1.2
Hemicellulose	10.5 ± 0.83
Phenols	18.7 ± 1.5
Extractable in solvent	3.1 ± 0.04
Ash	35.5 ± 1.9

2.2. Fungal Strain and Culture Conditions

Trametes hirsuta strain Bm-2 (GQ280373) was isolated from decaying wood in Yucatan, Mexico [9], and used throughout this work. The strain was maintained by periodical subculturing on plates with 2% malt extract and 2% bacteriological agar. A mycelia suspension of *T. hirsuta* was obtained by inoculating four 1-cm diameter plugs in a 250 mL Erlenmeyer flask containing Kirk's liquid basal medium, pH 6, which consisted of 10 g glucose, 5 g ammonium tartrate, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g KH_2PO_4 , 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 mg Thiamine, and 10 mL trace compound solution, without Tween 20 [10,11]. The flask was incubated at 35 °C, 150 rpm for 3 days. The biomass obtained was homogenized with the T18 digital ULTRA-TURRAX® by IKA®. The resulting suspension was used as inoculum in this work.

2.3. Fungal Broth Production (Fb)

T. hirsuta broth was produced in the YMPG medium whose composition ($\text{g} \cdot \text{L}^{-1}$) was as follows: Yeast extract 2 g, Malt extract 10 g, Peptone 2 g, Glucose 10 g, KH_2PO_4 2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 50 g, Thiamine hydrochloride 1 mg, Asparagine 1 g and wheat bran 2%. The pH was adjusted to 4.5 with HCl. 50 mL of the medium was transferred to 250 mL flasks and sterilized at 121 °C for 15 min in an autoclave. One mL of fungus inoculum was added in each flask and incubated at 35 °C, 150 rpm for 8 days. The liquid cultures obtained after fungal biomass separation were spectrophotometrically analyzed for enzyme activity at 25 °C. Laccase activity was determined using the substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, Sigma-Aldrich) (ABTS) and was measured at 420 nm ($\epsilon_{\text{max}} = 36,000 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) [12]. One unit of laccase activity was defined as the amount necessary to catalyze the formation of 1 mmol of oxidized ABTS min^{-1} .

2.4. Biologic Pretreatment

Two biological pretreatments were carried out. In both pretreatments, 10 g of the dry macroalgae consortium (Mc) were suspended in 100 mL of water (10% w/v). The pH of the Mc suspension was adjusted to 5 with an acid solution of HCl (1 N).

In the first case suspended Mc were inoculated with 5 mL of a mycelial suspension of *T. hirsuta* (McF), obtained as described in Section 2.3 and incubated at 35 °C, 150 rpm in an orbital shaker for 6 days prior to BMP tests. In the second case, the Mc suspension was inoculated with 10 mL fungal broth (Fb) with laccase activity of 7000 $\text{U} \cdot \text{mL}^{-1}$, obtained as described in Section 2.4. The resultant suspension (McFb) was incubated at 40 °C, 150 rpm for 24 h in an orbital shaker. Both pretreatments were assayed in triplicate.

2.5. Fourier Transforms Infrared (FT-IR) Analysis

Changes in the functional groups of untreated macroalgae and after biological and enzymatic pretreatment were observed using a Bruker spectrophotometer FT-IR Tensor II model (Milton, ON, Canada). Samples were analyzed in Platinum ATR (Attenuated Total Reflection) and spectra were obtained in the region of 4000–500 cm^{-1} , at a resolution of 4 cm^{-1} with 32 scans.

2.6. Scanning Electron Microscopy (SEM)

To study the microscopic structure of the macroalgae and the effect of pretreatment, a scanning electron microscope (SEM, model JSM-6360LV, JEOL, Tokyo, Japan) was used. Flour samples were mounted on a metallic stub using double-sided adhesive tape coated with a 15-nm gold layer and observed at 20 kV.

2.7. Biochemical Methane Potential (BMP) Tests

The BMP tests followed protocols and calculation in accordance with Valero et al. [13]. After biological pretreatment of macroalgae, BMP tests were carried out in 115 mL triplicate serum bottles capped with rubber septum sleeve stoppers, with a useful volume of 55 mL and a headspace volume of 60 mL. The inoculum consisted of a native mixed microbial consortium containing 30 g/L of deep soil, 300 g/L of cattle manure, 150 g/L of pig manure, 1.5 g/L of commercial Na₂CO₃, 5 g/L of sugar, and 1000 mL of tap water as described [14]. Previous to the BMP test, the inoculum was degassed for five days at 38 °C. The inoculum/substrate ratio was 2.05 g VS inoculum/g VS substrate for the three trials set-up: macroalgae consortium (Mc), macroalgae consortium + Fungal broth production (McFb), macroalgae consortium + *T. hirsuta* (McF).

Furthermore, the bottles were filled with distilled water up to 55 mL. Nitrogen was flushed to remove the air in the headspace and they were placed in an incubator at 38 °C for a period of 29 days. All the reactors were manually agitated once a day. Biogas production was measured by the pressure increase in the headspace volume. After each measurement, the reactors were vented until atmospheric pressure in the reactor was reached. Three blanks with 55 g of inoculum were also tested to measure the methane potential of the inoculum.

2.8. Analytical Methods

A digital pressure transducer with silicon measuring cell (ifm, type PN78, up to 2000 mbar, Essen, Germany)—was used to measure headspace pressure of the reactors through the septum with a connected syringe to the transducer. Biogas characterization was determined on a Molesieve column (30 m long, 0.53 mm internal diameter and 0.25 µm film thickness) in a gas chromatograph (Clarus 500-Perkin Elmer, Waltham, MA, USA) with the thermal conductivity detector (TCD), nitrogen was used as gas carrier and temperatures of 75, 30 and 200 °C for the injector, oven and detector respectively. Conductivity, temperature, pH and total solids (TS), volatile solids (VS) were analyzed following standard methods [15], colorimetric methods (Hach Company DR-890, Loveland, CO, USA) was used to determinate chemical oxygen demand (COD).

2.9. Statistical Analysis

All measurements on the physicochemical parameters were performed in triplicate. Results were expressed as means ± standard deviation (SD) subtracting methane production from the inoculum using Statistica 9 software.

2.10. Numerical Calculations

The accumulated volumes of methane were calculated by the cumulative summation of methane volumes in accordance with Valero et al. [13]

3. Results and Discussion

3.1. Biological Pretreatment

The fungal broth, obtained from the growth of *T. hirsuta* in a 2% wheat-bran-enriched Kirk medium, showed an extracellular laccase activity of 7000 U·mL⁻¹ much higher than the value (170 U·mL⁻¹ at four days) reported by Hom-Diaz et al. [8], for *T. versicolor* grown in Kirk medium.

Our results demonstrate that wheat bran (lignocellulosic substrate) is an efficient inducer for the production of this enzyme at an extracellular level. Previous works have shown that the Bm-2 strain has a battery of laccase genes, which are differentially expressed when different phenolic compounds are present in the lignin structure [7]. Zapata-Castillo et al. [16] reported that *T. hirsuta* Bm-2 produces three laccase isoenzymes (Lac I, II, III) on wheat bran. These three share some properties, but are different in other characteristics. They are very tolerant to temperature and organic solvents, which underlines their significant role in a variety of processes.

The fungal broth produced by *T. hirsuta* culture in Kirk's medium with wheat bran 2% is mostly rich in laccase enzymes among other enzymes. Sing et al., 2013 [17] observed the secretion of other enzymes such as cellulases and hemicellulases after three days cultivation of *T. versicolor*. This is very important for macroalgae consortium cell wall degradation. These results offer a new direction for biotechnological processes using mixtures or combinations of oxidizing enzymes for biological pretreatment in order to improve biomethane production.

3.2. FT-IR and SEM Analysis Biology Pretreatment

Figure 1 shows the FT-IR spectral of the macroalgae consortium (Mc), both untreated and after biological pretreatment with fungi (McF) from strain *T. hirsuta* and fungal broth (McFb), which presented typical bands of major lignocellulosic biomass components.

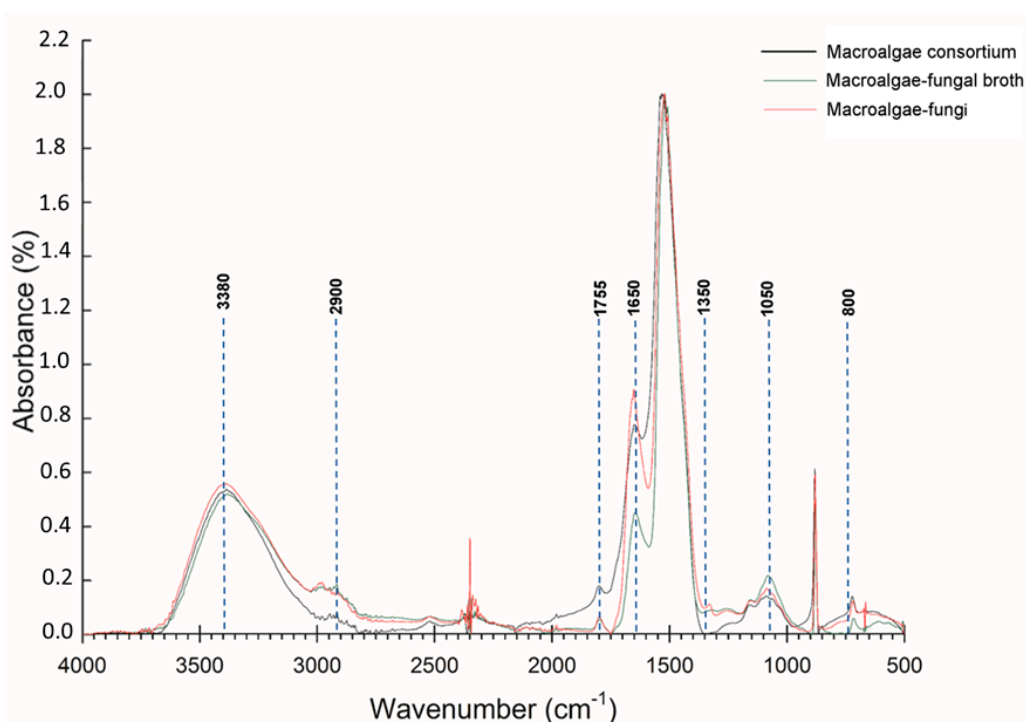


Figure 1. FT-IR spectral of untreated and pretreated macroalgae consortium sample. Black line: macroalgae consortium (Mc), green line: macroalgae–fungal broth (McFb) and red line: macroalgae fungi (McF).

The FT-IR spectral data display a strong, broad absorption band around 3380 cm^{-1} , which arises from the stretching of -OH groups. This band may be associated with the hydroxyl groups of cellulose, hemicellulose and lignin. These findings coincide with previously reported results for sugarcane bagasse submitted to a pretreatment process with steam explosion and sodium hydroxide [18–20].

An increase in signals for C–H stretching at approximately 2900 cm^{-1} and 1350 cm^{-1} was observed in the treatments with the fungi (McF) and fungal broth (McFb), which can be attributed to

exposure of the amorphous and crystalline cellulose after the lignin has been solubilized. It coincides with the proposal of Nelson and O'Conner [21] on a method for calculating the "Total Crystalline Index" (TCI) as the ratio of absorption intensities from 1372 cm^{-1} to 2900 cm^{-1} . C–O–C stretching that is typical of xylans of the hemicellulose is believed to be responsible for the sharp band at 1050 cm^{-1} [19]. This confirms the delignification of the macroalgae consortium in the biologic treatment.

The lower band intensity at 1755 cm^{-1} , which can be seen in the spectrums of McF and McFb, is due to the fact that delignification of the biomass promotes a breakdown of the carbonyl ester bonds between hemicellulose and lignin. Laccase enzymes catalyze this delignification, which has been the subject of much research showing it to be effective both for the removal and/or the modification of the lignin polymer, and for the reduction of the phenolic content of pretreated lignocellulosic materials [22]. In general, the absorption spectral bands at 3380 , 2900 , 1350 , 1050 , and 800 cm^{-1} are associated with cellulose and hemicellulose core structures, as described by Ju et al. [19] in the chemical treatment of sugarcane bagasse.

3.3. SEM Analysis of Untreated and Pretreated Macroalgae Consortium

Generally, an efficient pretreatment strategy includes the removal of the cross-linked matrix of lignin and hemicelluloses, and an increase in the porosity and surface area of cellulose, for later enzymatic hydrolysis. In the scanning electron micrographs presented in Figure 2, the compact structure of lignocellulosic biomass of the macroalgae consortium before pretreatment can be observed (Figure 2a'), as can the structural alterations that took place on the surface of fibers subsequent to biological pretreatment with fungal broth (McFb; Figure 2b') and fungi (McF; Figure 2c') from the *T. hirsuta* strain.

In Figure 2a', a smooth and compact surface of the epidermis of untreated cells can be seen, whereas in the treatment with the enzymatic extract (Figure 2b') it is possible to observe a degradation of the fiber at the superficial level, which may be linked to the fact that the action of the laccases removed the lignin only partially [22].

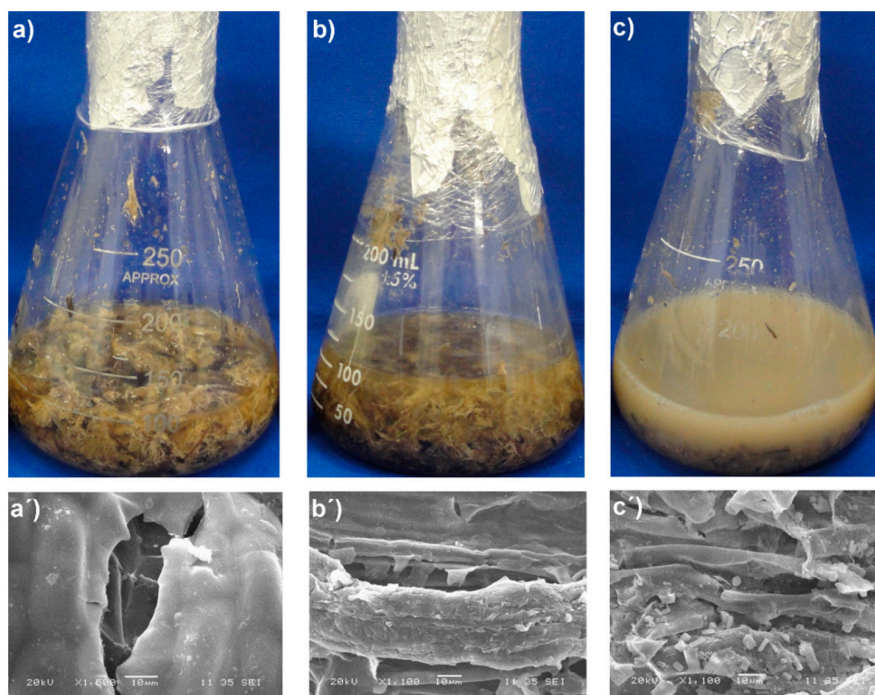


Figure 2. Scanning electron micrographs of macroalgae consortium samples before and after pretreatment. (a,a'): macroalgae consortium (Mc), (b,b'): macroalgae + fungal broth (McFb), (c,c'): macroalgae + fungi (McF).

Treatment with Bm-2 strain (*T. hirsuta*) (Figure 2c) promoted the formation of grooves on the surface of the material. These were probably a result of the lignocellulose fiber being completely destroyed, and the hemicellulose partially removed. This in turn is probably the result of the presence of other enzymes and other mediators generated by *T. hirsuta* during its culture, and these could also play a part in the solubilization of macroalgae consortium biomass.

3.4. Production of Biogas in BMP Test

Macroalgae Consortia and BMP Tests

For the purposes of evaluating the increase in anaerobic biodegradability in BMP tests, the experiment was extended over 29 days, until accumulated production of methane leveled off as shown in Figure 3.

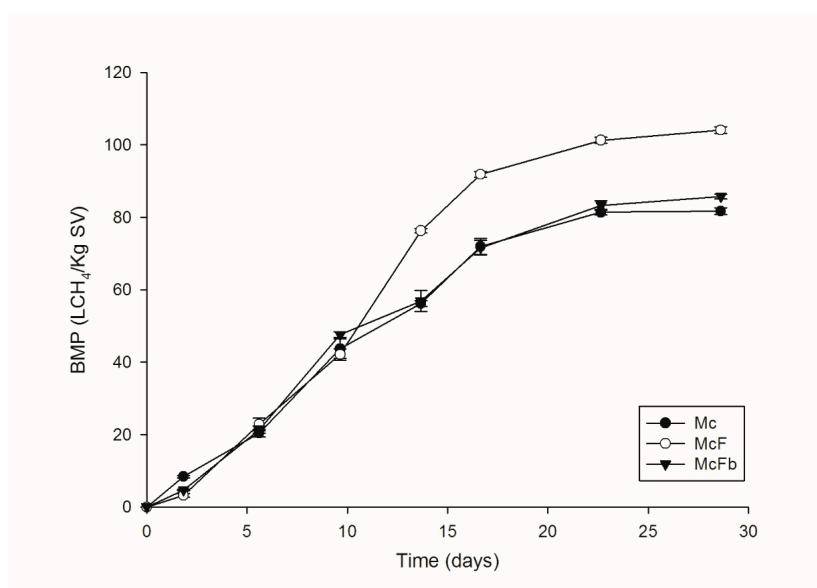


Figure 3. Accumulated production of methane for the anaerobic digestion of macroalgae consortium employing two pretreatments and a control. Macroalgae consortium (Mc); macroalgae consortium + fungal broth (McFb); macroalgae consortium + fungi (McF).

As can be seen from the results (Table 2), the macroalgae consortium + fungi trial produced the highest methane yield as compared to non-pretreated macroalgae consortium and macroalgae consortium + enzymatic Broth.

Table 2. Methane content and production for the various BMP test trials.

Trial	Methane Content (%)	BMP L CH ₄ ·kg VS ⁻¹
Macroalgae consortium	40 ± 1.0	81 ± 1.2
Macroalgae consortium + enzymatic broth	46 ± 0.5	86 ± 0.7
Macroalgae consortium + fungi	52 ± 1.0	104 ± 1.4

The macroalgae consortium used in this work was characterized by a $15.6 \pm 0.51\%$ of lignin, in accordance with Carrere et al. [23] lignin, cellulose, hemicelluloses, total uronic acids, proteins and soluble sugars content, as well as crystallinity of lignocellulosic substrates, were used to identify the most influential parameters linked to the biomethane potential values, the most important parameter being the lignin content (above 12%), which was negatively correlated to the BMP. Likewise, the large

amount of ash in the macroalgae is linked to salt. Table 1 shows as that the ash content was high of $35.5\% \pm 1.9$ with a biodegradability of 0.35 ± 0.02 . Furthermore, this was observed in SEM analysis (Figure 4). Thus the buildup of salt in the digester over time may be a further factor in achieving long term anaerobic digestion, biodegradability and biomethane yields [5].

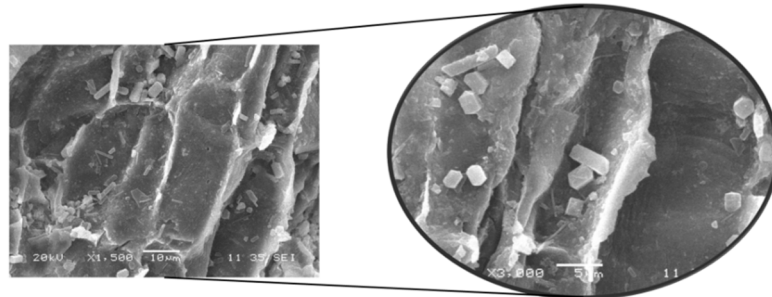


Figure 4. Content of salts, mainly sodium chloride and potassium chloride, in the macroalgae consortium.

Figure 2 (Figure 2a,b), shows how the feedstock had a lignocellulosic appearance, and it was evident, as in Figure 2c) that biological pretreatment with macroalgae consortium + fungi, which obtained $104 \text{ L CH}_4 \cdot \text{kg VS}^{-1}$, was higher to macroalgae consortium + enzymatic broth and macroalgae consortium, whose values were 85 and $82 \text{ L CH}_4 \cdot \text{kg VS}^{-1}$ respectively.

Furthermore, the C:N ratio of macroalgae consortium harvested in Progreso was 20:5 (in January), a value which is within the ideal range for anaerobic digestion (20–30:1). Adams et al. [24] and Oliveira et al. [25] found that at the beginning of the year, the macroalgae contained the lowest quantities of carbohydrate and the highest of ash and metals, whereas samples collected in July had the highest carbohydrate content, and the lowest quantity of metals and ash. Ross et al. [26] asserted that as the macroalgae progresses through its growth cycle, the biochemical composition alters. In March, the macroalgae normally have a high level of protein and alginic acid, and a low level of carbohydrate. During the spring the sugar content (e.g., mannitol) rises as photosynthesis increases, and there is a correlative drop in ash, proteins and alginic acid. These results also coincide with those of Tabassum et al. [5], who mentioned that *Fucus vesiculosus* and *Fucus serratus* harvested in March and subjected to biologic pretreatment had values of $126 \text{ L CH}_4 \cdot \text{kg VS}^{-1}$ and $101 \text{ L CH}_4 \cdot \text{kg VS}^{-1}$ respectively. Likewise the carbohydrate values were on average 31%, whereas in the macroalgae consortium in this study they were 22%. The carbohydrate content (alginic acid) in the seaweed or macroalgae is a significant parameter in the BMP; higher amounts produce higher BMPs [5,27].

The content of phenols in the macroalgae consortium was 19%, and these results are in agreement with Allen et al. [27], who stated that high levels of phenols (up to 14%) are natural inhibitors of the anaerobic digestion process, and a BMP yield of $166.3 \text{ L CH}_4 \cdot \text{kg VS}^{-1}$ and $110 \text{ L CH}_4 \cdot \text{kg VS}^{-1}$ was reported in Ireland and Norway [27,28], respectively, where a high level of phenol gives a low index of biodegradability and low kinetic decay values. Therefore, macroalgae consortiums are very good candidates for pretreatment processes and evaluation of variation depending on the season, because several shown that the level of polyphenols in seaweed or macroalgae varies during the year [5,29,30]. And as Tabassum et al. [5] mentions in his article, further investigation is necessary to assess the optimum month for harvesting macroalgae consortiums and a suitable pretreatment to improve their potential for biomethane.

The metals contents can be seen in Table 3, of the eight elements which were found, those showing the highest concentrations were Mg, K and Na. These results agree with Adams et al. [24]. Elements existing at comparatively elevated concentrations in macroalgae can also be poisonous or problematic when released as volatiles. Likewise, in this study the value of Zn was 60.1 and Adams et al. [24] found that Zn content in *Fucus vesiculosus* of 358 ppm contrasts to the 30 ppm detected in *L. digitata*. The size

of the difference makes it improbable that it indicates simply a difference in the ability of the species to accumulate Zn.

Table 3. Methane content and production for the different Biochemical Methane Potential (BMP) test trials.

Component Name	Average (ppm)
Fe	584.9 ± 3
Mn	12.9 ± 2
Cu	7.5 ± 0.2
Zn	60.1 ± 0.1
Na	45,210 ± 25
K	28,500 ± 55
Cd	<0.1
Mg	4806.1 ± 20

In this study, the macroalgae consortium contains macrominerals such as Na, K, and Mg, and trace elements like Fe, Cu, Zn and Mn. These metals occurring in biomass species are significant in conversion processes because they have an effect on slagging, fouling and other problems linked to ash [26]. The sum of the alkali metals Ca, K, Mg, Na was 78 g·L⁻¹. This value was similar to that obtained by Ross et al. [26] in the first months of the year (98 ± 5 g·L⁻¹). Knowing the potassium and sodium content permits predicting serious problems in the possible use of this fuel in combustion or gas production. It likewise provides an estimate of the fouling potential of the ash [26].

4. Conclusions

The availability of macroalgae consortium waste used in this study, is promising for biomethane production, and it is a readily available resource that in the future, could meet goals for advanced biofuels in the Mexican Caribbean zone. Likewise, the effect of Bm-2 strain on macroalgae consortiums as a pretreatment step before proceeding to anaerobic digestion, is promising for the future scaling in continuous reactors. The enhancement of biogas production that reached 104 L CH₄·kg VS⁻¹, was statistically higher than values obtained with enzymatic pretreatments. Thus, the process we propose in this study can play a role in providing a solution to a problem currently faced by Mexican beaches, and contribute to providing clean, sustainable energy.

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Conflicts of Interest: The authors declare no conflicts of interest.

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