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SARGASSUM-PIG MANURE CO-DIGESTION SYNERGISTIC EFFECT ON BIOCHEMICAL METHANE POTENTIAL

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SARGASSUM-PIG MANURE CO-DIGESTION SYNERGISTIC EFFECT ON BIOCHEMICAL METHANE POTENTIAL

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DEDICATION

Julio Cortázar, quite rightly, said that "words are never enough when what needs to be said overflows the soul"; and that is exactly what happens in the following lines.

I want to dedicate this work to my family, especially to my mother and sister, who have always been motivating me, supporting me and sharing every moment of my life, whether satisfactory or adverse.

To my friends, those who have been with me for many years and whom I had the fortune to meet during this stage, especially to Anita for her company, advice and affection; to Denisse, for her support in my experimental phase and personally, for the laughter and advice; to Fanny, for her teachings, comforting hugs, advice and all her affection; to Erika de la Vega, not only for her support in the laboratory but also for her company, her time and for listening to me when I needed it; to Juan Carlos Herrera also for his support in the laboratory; to Thali for her advice and blessings; to Miriam, for the pleasant conversations and good advice; to Danni, for being my friend since bachelor's and now in the master's program, for continuing to support and comfort me; to Migue for being there since bachelor's, and even before without being classmates; and the last days of the battle, to the thesis students of the Bioprocesses lab, especially to Jessi and Angie for the laughs and for making me feel very proud of their learning process.

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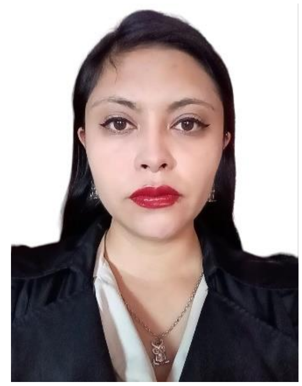
BIOGRAPHY



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RESUMEN GENERAL

EFECTO SINÉRGICO DE LA CODIGESTIÓN DE SARGAZO Y ESTIÉRCOL DE CERDO EN EL POTENCIAL BIOQUÍMICO DE METANO

La acumulación de sargazo en las playas del Caribe mexicano es un problema que afecta a varios sectores de la población, en términos de salud y economía. Una vez recolectada esta macroalga, es necesario tomar medidas para que su eliminación no genere más efectos negativos en el medio ambiente. La digestión anaeróbica representa una alternativa de uso de esta biomasa. El objetivo de este estudio fue elucidar la viabilidad del uso de *Sargassum fluitans* y *Sargassum natans* en codigestión con estiércol de cerdo. Se realizaron cinco tratamientos con diferentes proporciones de sustrato (100S-0PM, 65S-35PM, 50S-50PM, 30S-70PM y 0S-100PM). Los resultados mostraron un efecto sinérgico significativo que mejoró el PBM de 79.5 a 160.4% con respecto a las monodigestiones. El mayor PBM fue de 441.47 $mLCH_4{\cdot}g^{-1}{}_{VSFed}$, que corresponde al tratamiento 50S-50PM con una relación C:N de 16.8. Los resultados de este estudio demostraron que la codigestión de biomasa de sargazo con estiércol de cerdo aumentó el rendimiento de metano.

Palabras clave: Sargazo, codigestión, acumulación de AGV, efecto sinérgico.

GENERAL ABSTRACT

SARGASSUM-PIG MANURE CO-DIGESTION SYNERGISTIC EFFECT ON BIOCHEMICAL METHANE POTENTIAL

The accumulation of sargassum on the beaches of the Mexican Caribbean is a problem that affects various sectors of the population, in terms of health and the economy. Once it has been collected, it is necessary to take measures so that its disposal does not generate more negative environmental effects. Anaerobic digestion represents an alternative use of this biomass. The objective of this study was to elucidate the feasibility of using *Sargassum fluitans* and *Sargassum natans* in co-digestion with pig manure. Five treatments were carried out with different substrate ratios (100S-0PM, 65S-35PM, 50S-50PM, 30S-70PM and 0S-100PM). The results showed a significant synergistic effect improving BMP from 79.5 to 160.4% with respect to mono-digestions. The highest BMP was 441.47 mL $CH_4 \cdot g^{-1}_{VSFed}$, which corresponds to 50S-50PM treatment having a C:N ratio of 16.8. Results of this study demonstrated that co-digestion of sargassum biomass with pig manure increased the methane yield.

***Keywords*:** Sargassum, co-digestion, VFA accumulation, synergistic effect.

1. GENERAL INTRODUCTION

Anaerobic digestion (AD) is a process by which a consortium of microorganisms, mainly bacteria and archaea, degrade available organic matter to produce biogas, composed mainly of methane (CH_4) and carbon dioxide (CO_2). The importance of this technique lies in the fact that it is a natural process whose objective is to provide renewable energy, since, generally, the substrate or substrates used are considered waste. Thus, through AD, the organic matter content is used before disposal, and part of the remaining waste can be used as biofertilizer (Liebetrau et al. 2017).

The substrates to be used in this process generally depend on the area where it is carried out; for example, in the Yucatan Peninsula, there are mainly two waste products that generate significant environmental impacts: sargassum and pig manure.

The genus Sargassum contains more than 350 species, of which *S. natans* and *S. fluitans* have been identified as the cause of the denominated "golden tide" in the Mexican Caribbean. These species are holopelagic and have a vegetative reproduction process and do not physically connect to the ocean floor during their life cycle (Smetacek and Zingone, 2013). The accumulation of this macroalgae causes diverse affectations to the coastal zones, mainly related to its decomposition, which generates hydrogen sulfide, methane and leachates, resulting in harmful effects to human health, death of marine flora and fauna, contamination by toxic elements such as arsenic, unpleasant odors and a considerable decrease in the influx of tourists to the beaches that are affected by the excessive arrival of Sargassum (Chávez et al., 2020).

On the other hand, since 2015, Yucatan ranks fifth in the country in pig farming, being the state that provides 9% of the national production. This industry is growing at an annual rate of 4.5%, which is higher than that of Sonora (2.6%) and Jalisco (1.7%), the main pork producers in Mexico (Calderón-Mólgora et al*.,* 2021). This represents an obvious economic benefit; however, other sectors have been highly affected; such is the case of the environment through groundwater contamination, air pollution and soil erosion (Cheng et al., 2020). Batllori-Sampedro (2016) mentions that, in the Registro Público de Derechos de Agua of the Conagua, at the end of 2013, 374 livestock discharges were generated in the states of the Yucatan Peninsula, with a total of 9 million cubic meters per year, of which 77 % corresponded to Yucatan, 17 % to Campeche and 6 % to Quintana Roo. The situation is aggravated by the difficulty of not having sanitary drainage in the region (due to the hardness of the soil and the flatness of the terrain), causing groundwater quality to deteriorate (Calderón-Mólgora et al., 2021).

In this perspective, anaerobic digestion is emerging as an alternative solution to these two problems in the Yucatan Peninsula, which also allows taking advantage of this biomass to produce biofuels, in this case, biogas.

It has been reported that the use of sargassum is not feasible for large-scale energy production (Thompson et al. 2021), therefore, it is suggested to use pretreatments to improve the biodegradability of the biomass, or to implement a co-digestion system. The advantages of implementing co-digestion include improving the nutrient imbalance of the substrates to be used, modifying the C:N ratio to an optimal range (20:1-30:1), as well as increasing the buffering capacity of high NH_3 and sulfur concentrations for better degradation and more stable biogas production (Zhang et al., 2013) and contribute to rapid microbial growth and regulate microbial activity during anaerobic digestion (Espinosa-Solares et al., 2022).

Therefore, the purpose of this research is to elucidate the feasibility of using sargassum in co-digestion with pig manure by evaluating its biochemical methane potential (BMP).

2. LITERATURE REVIEW

2.1 Anaerobic digestion of sargassum

After the excessive accumulation of this macroalgae in the different coastal areas of the planet, which has been present since 2011 (Wang & Hu, 2016), some researchers were interested in finding alternative treatments for this biomass in order to reduce the environmental impact it generates. The following is a chronological account of these studies. Most of these works have focused on using pretreatments to reduce the amount of recalcitrant compounds and, thus, increase the biodegradability of the biomass.

Such is the case of Jard et al. (2013) who worked with a selection of ten macroalgae obtained from the French coast of Brittany, among which are *Sargassum muticum,* they performed the chemical characterization concerning carbohydrates, proteins, fiber, and lipids and determined the presence and characteristics of value-added molecules such as polyphenols and alginates; finally, they analyzed their BMP. For the latter, crude biomass was used; each treatment contained 2 g_{VS} and 2 g of inoculum, which was anaerobic sludge from wastewater from a sugar industry. The experiment was carried out in batch mode for 40 days at 35 °C, and a yield of 130 mL $CH_4 \cdot g^{-1}_{VS}$ was obtained.

Costa et al. (2015) used *Sargassum* sp., collected in the north coastline of Portugal, in a process of dark hydrogen fermentation with *Caldicellulosiruptor saccharolyticus* and subsequent anaerobic digestion for 42 days. They used biomass pretreated in autoclave at 121 °C and 1 bar for 15 minutes, using 2.5, 4.9 and 7.4 $g_{VS} \cdot L^{-1}$ of biomass, for which yields of 541 mL $CH_4 \cdot g^{-1}_{VS}$, 345 mL $CH_4 \cdot g^{-1}_{VS}$ and 281 mL $CH_4 \cdot g^{-1}_{VS}$, were obtained, respectively. The experiment was performed in batch and granular sludge from a brewery industry was used as inoculum.

Soto et al. (2015) carried out an analysis of the BMP of *S. muticum* collected from the coast of Coruña, Spain; without pretreatment and sampled at different times of the year. They used anaerobic sludge from a sea fish canning wastewater as inoculum and biomass concentration was 5 and 10 g_{TS}·L^{-1}, with which they obtained yields ranging from 166 to 208 mL CH_4·g^{-1}_{VS}.

Milledge and Harvey (2016) worked with *S. muticum* collected from Walpole Bay, Margate, England. They used raw biomass, ensilage whole and chopped prior to ensiling; each treatment using 1 g_{VS} and anaerobic sludge from paper making liquid waste as inoculum. The experiment was incubated at 37 °C for 28 days. The best yield was reported for the whole ensilage treatment (110 mL CH_4·g^{-1}_{VS}), followed by raw biomass (100 mL CH_4·g^{-1}_{VS}) and chopped prior ensiling (60 mL CH_4·g^{-1}_{VS}).

Milledge et al. (2018) evaluated the effect of fresh water washing on biogas production with *S. muticum* collected from Minis Bay, Kent, England. It was found that, after 28 days, methane production between the two treatments was not statistically significant (177 mL CH_4·g^{-1}_{VS} for washed biomass and 225 mL CH_4·g^{-1}_{VS} unwashed biomass); however, washing delayed methane production. The experiment was conducted at 37 °C and employed granular sludge from a paper making liquid waste as inoculum.

Tapia-Tussell et al. (2018) utilized *Sargassum* sp. from the beaches of Progreso, Yucatan, Mexico. The objective of their study was to evaluate biogas production from biomass biologically pretreated with a Bm-2 strain (*Trametes hirsuta*) isolated from decaying wood. Two biological treatments were performed; in the first case (McF), macroalgae previously suspended in water were inoculated with 5 mL of a mycelial suspension of *T. hirsuta.* and incubated at 35 °C, with constant agitation for 6 days prior to BMP tests. For the second case (McFb), the suspension was inoculated with 10 mL of fungal broth and incubated at 40 °C with constant agitation for 24 h. Additionally, BMP assay for crude biomass (Mc) was performed. The experiments were incubated at 38 °C for 29 days, at the end of which, the highest yield was reported for McF (104 mL CH_4·g^{-1}_{VS}) which improved by 20% over Mc (81 mL CH_4·g^{-1}_{VS}). A native mixed microbial consortium adapted to degrade cattle manure and pig manure was used as inoculum.

Milledge et al. (2020) carried out a BMP analysis of *S. natans VII, S. natans I* and *S. fluitans* species, as well as with a freeze-dried mixture and a fresh mixture of the same. This biomass was not subjected to any pretreatment. The macroalgae were collected from Shark Bay, South Caicos, Turks and Caicos. The experiment was conducted for 28 days and granular sludge was used as inoculum. In addition, proximate analysis, determination of metal content, metalloids and phenolic compounds were carried out. The BMP obtained was 145 mL $CH_4 \cdot g^{-1}_{VS}$ for *S. natans VII,* 66 mL $CH_4 \cdot g^{-1}_{VS}$ for *S. natans I* and 113 mL $CH_4 \cdot g^{-1}_{VS}$ for *S. fluitans*. Both sargassum mixtures did not produce methane, which was attributed to the high levels of phenolic compounds, because a strong correlation was found among BMP and phenolic content. Considerable differences were also found in the mixed composition of sargassum and individual species, being higher in ash, calcium, iron and arsenic.

Thompson et al. (2020) carried out a hydrothermal pretreatment of a mixture of *S. natans* and *S. fluitans* collected from Conset Bay, Barbados. The experiment lasted 21 days at 35 °C using anaerobic digestate from wastewater treatment as inoculum. The highest methane yield obtained was 116.72 mL $CH_4 \cdot g^{-1}_{VS}$. The authors report that there is no linear relationship between increased solubilization and biogas productivity and attribute the low methane yield to the formation of Maillard reaction products and inhibitory compounds during hydrothermal pretreatment.

Flórez-Fernández et al. (2021) used *S. muticum* collected from Praia da Mourisca, Pontevedra, Spain which they subjected to pressure treatment and autohydrolysis as well as to subsequently compare the BMP with that of raw biomass. Anaerobic sludge from a domestic wastewater and a full-scale plant treating fish canning wastewater were used as inoculum. All treatments used 3 g_{TS}. A yield of 170 mL $CH_4 \cdot g^{-1}_{VS}$ was obtained for the pretreated biomass and 80 mL $CH_4 \cdot g^{-1}_{VS}$ for the raw biomass.

Rezaei et al. (2021) worked with *Sargassum* sp. collected from the shores of southeast Iran (Chabahar port). For the BMP experiment, digestate from a laboratory scale digester fed with cow manure as inoculum was used. The experimental units were incubated at 37 °C, all with 26 g of seaweed residual and five levels of sludge from a drinking water treatment plant (DWTS): 0, 2, 6, 12 and 18 mg·L^{-1}. The best yield obtained was 199 mL CH_4·g^{-1}$_{VS}$ from the treatment with 6 mg·L^{-1} of DWTS, which was 30% better than the control (152 mL CH_4·g^{-1}$_{VS}$).

Abomohra et al. (2021) analyzed the feasibility of using three species of macroalgae; *Ulva* spp. phylum Chlorophyta, *Gracilaria* spp. of the phylum Rhodophyta and *Sargassum* spp. of the phylum Phaeophyte, for heavy metal biosorption and biofuel production. The algae were collected from the coastal area ok Jizan City, Kingdom Saudi, Arabia. Sargassum showed the highest cumulative biosorption of copper (Cu^{2+}) with a removal efficiency of 80%. Subsequently, a BMP experiment was conducted with raw *Sargassum* spp. (RB), biomass adsorbed heavy metals (BHM), residual fermented RB (FRB) and residual fermented BHM (FHBM). Anaerobic sludge was used as inoculum, the experimental units were incubated at 37 °C for 23 days. All treatments had 5% TS. The methane yields obtained were: 172.7 mL CH_4·g^{-1}$_{VS}$ for RB, 158.3 mL CH_4·g^{-1}$_{VS}$ for BHM, 150.1 mL CH_4·g^{-1}$_{VS}$ for FRB and 166.6 mL CH_4·g^{-1}$_{VS}$ for FBHM. It is important to stress that the presence of Cu^{2+} in BHM showed a significant reduction in methane yields by 5.2%.

Wahab et al*.* (2021) evaluated the methane production of *S. polycystum* obtained as a dry milled from a commercial supplier in Galway, Ireland. The BMP test was carried out during approximately two months at 37 °C. Industrial sludge from food waste was used as inoculum. The BMP reported was 226 mL CH_4·g^{-1}$_{VS.}$

Maneein et al*.* (2021) studied the effect of seasonality and freshwater flushing on methane production using *S. muticum* as substrate. The macroalgae collected in spring were taken from Coast, Ramsgate, UK and summer macroalgae was collected in Broadstairs, UK. The inoculum was taken from an anaerobic digester treating paper making waste. The yields obtained after 36 days of digestion were 139. 7 mL CH_4·g^{-1}$_{VS}$ and 128.2 mL CH_4·g^{-1}$_{VS}$ for unwashed biomass collected in spring and summer, respectively, and 163.2 mL $CH_4 \cdot g^{-1}_{VS}$ y 170.7 mL $CH_4 \cdot g^{-1}_{VS}$ for washed biomass collected in summer, respectively.

Farghali et al. (2021) used *S. fulvellum* from commercial aquaculture farms along Indian Ocean and digester slurry collected from a mesophilic biogas plant as inoculum. Their study analyzed the impact of mechanical, chemical and enzymatic treatments on biogas generation. The mechanical treatment (M_{red}) consisted on particle size reduction. Chemical treatments were carried out with 2M HCl at concentrations of 0.36 $mL \cdot g^{-1}$ (M_{acid1}) and 0.18 $mL \cdot g^{-1}$ (M_{acid2}) as well as with 6M NaOH at concentrations of 0.09 $mL \cdot g^{-1}$ ($M_{alkali1}$) and 0.04 $mL \cdot g^{-1}$ ($M_{alkali2}$). For biological treatment (M_{enz}) the enzyme cellulase Viscamyl™ Flow (0.01 $mL \cdot g^{-1}$) was used. In addition, biomass without particle size reduction was emplyed as a control (M_{raw}). The experiment lasted for 40 days at a temperature of 55 °C. The treatment with which the highest yield was obtained was M_{enz}, which produced 186.60 mL $CH_4 \cdot g^{-1}_{VS,}$ resulting in an increase of 116.64% and 33.48% over M_{red} and M_{raw}. On the other hand, the chemical treatments improved methane yield by a range of 6.53% to 45.65% compared to M_{red}. Furthermore, the authors stressed that it is more advisable to use raw biomass with cellulase enzyme supplementation, considering that M_{raw} reported a higher yield (145.69 mL $CH_4 \cdot g^{-1}_{VS}$) compared to the chemical and mechanical treatments; this guarantees a sustainable use of this macroalgae.

Yuhendra et al. (2021) worked with *S. fulvellum* from commercial farms in Indonesia. They carried out mechanical pretreatment by particle size reduction (Sr), then used 20 g of dried macroalgae + 180 mL of water as a control for chemical treatments. These were carried out as follows: 40 mL of 2 M HCl to 1 L of macroalgae (Sac1), 20 mL of 2 M HCl to 1 L of macroalgae (Sac2), 10 mL of 6 N NaOH to 1 L of macroalgae (Sal1), and 5 mL of 6 N NaOH to 1 L of macroalgae (Sal2). As well, the enzymatic treatment (Se) had as control 20 g of dried macroalgae of original size + 180 mL of water (So), this was performed by adding 1 mL of cellulase Viscamyl™ Flow to 1 L of macroalgae. As a result, it was found that particle size reduction increased the degradation rates of VS and VFA; the methane production rate increased by 52.34% with respect to So and by 9.83-18.26% with respect to the rest of the treatments. Authors suggest an inhibition of the anaerobic consortium in chemical and enzymatic treatments due to the decrease in methane production; thus, they suggest the use of small size macroalgae, since it is the one that allows to reach the maximum biological activity.

López-Aguilar et al*.* (2021) emplyed *Sargassum* sp. collected from the hotel area of Cancun, Quintana Roo, Mexico to carry out a batch experiment with anaerobic activate sludge from a wastewater treatment as inoculum in three treatments with different concentrations of VS as follows: D2 with 2.575% VS, D3 with 5.15% VS and D4 with 7.725% VS. The experimental units were incubated at 37 °C for 38 days. At the end of the experiment the yields obtained were: 348 mL $CH_4 \cdot g^{-1}_{VS}$ for D2, 319 mL $CH_4 \cdot g^{-1}_{VS}$ for D3 and 183 mL $CH_4 \cdot g^{-1}_{VS}$ for D4.

Thompson et al. (2021) worked with a mixture of *S. fluitans* and *S. natans* (PS) collected from coastal Waters of Conset Bay, Barbados. The inoculum for the experiment was anaerobic digestate from a wastewater treatment plant. Biomass was co-digested with Food Waste (FW). The treatments consisted of untreated PS and untreated FW, hydrothermally pretreated PS and untreated FW as well as pretreated PS and pretreated FW, all with three different weight ratios: 25:75, 50:50 and 75:25. The authors stressed the benefit of carrying out co-digestion of both substrates, mainly in the improvement of methane yield, buffering capacity of the system and redistribution of metal content. The pretreatment, on the other hand, enhanced the hydrolysis of the substrates by improving COD solubilization and acetic acid formation. The maximum cumulative yield of 292.18 mL $CH_4 \cdot g^{-1}_{VS}$ was obtained from co-digestion of pretreated PS and pretreated FW at a ratio of 75:25.

Finally, Ayala-Mercado et al*.* (2022) carried out a pretreatment by steam explosion and extrusion to increase digestibility of a mixture *S. fluitans* and *S. natans* collected from Puerto Morelos, Quintana Roo, Mexico. The biomass was subjected to a washing and dehydration process to reduce the salt content.

The BMP of treated and untreated sargassum was determined in batch tests at 36 °C. Granular anaerobic sludge from a local beverage producer was used as inoculum. Both treatments contained 1.8 g VS. Methane yield obtained for both treatments were not statistically different (114 $CH_4 \cdot g^{-1}{}_{VS}$ for steam explosion and 108 mL $CH_4 \cdot g^{-1}{}_{VS}$ for extrusion). Authors stressed that the extrusion process may be favored because it has an integrated dehydration effect. This preconditioning is necessary to remove dissolved inorganic solids outside the cell walls in the drained liquid and results in a more suitable biomass. It is also mentioned that biodigestion in the batch trial occurs under optimized conditions, where inhibition effects are suppressed due to the dilution effect and nutrients are sufficiently available due to an inoculum-substrate ratio of 2:1. Therefore, they suggest conducting further research on continuous anaerobic digestion that evaluates inhibition effects.

2.2 Anaerobic digestion of pig manure.

Regarding pig manure, numerous studies have been carried out to analyze the feasibility of using it as a substrate for anaerobic digestion. These works have been presented since 1983 and are still being reported today. For this substrate, the improvement of the final methane yield has been sought through pretreatments, addition of nanoparticles or other elements that reduce the inhibitory effect of the metal content in this manure or supplement the deficiency of some trace elements that are key factors for AD, such as Fe, Co, Ni, among others. Co-digestion of pig manure with other substrates, such as agro-industrial wastes, macroalgae, FW, grass, and others, has also been widely reported. Some of the most relevant works on pig manure AD in batch are described below, starting with those carried out in mono-digestion, followed by those realized by co-digestion.

Zhang et al. (2014) investigated the effect of pig growth stages on DA. Experiments were conducted using gestating pig manure (GSM), post-weaned piglet manure (SNM), growing fattening manure (GFM), and mixed manure (MM) at four different substrate concentrations: 40, 50, 65, and 80 $g_{VS}·L^{-1}$. Maximum methane yields for each growth stage were 354.7 mL $CH_4·g^{-1}_{VS}$ for MM, 328.7 mL $CH_4·g^{-1}_{VS}$ for SNM, 282.4 mL $CH_4·g^{-1}_{VS}$ for GSM y 263.5 mL $CH_4·g^{-1}_{VS}$ for GFM, all at the concentration of 40 $g_{VS}·L^{-1}$. Authors attribute the variability of the results to feeding strategies and nutrient digestibility at different growth stages.

Ferreira et al. (2014) employed thermal pretreatment with different combinations of temperature and time, between 150-180 °C and 5-60 min. The results indicated that methane yield and degradation rates of experimental units with pretreated biomass increased compared to untreated pig manure. The highest yield, 329 mL $CH_4·g^{-1}_{VS}$, was obtained for the 170 °C-30 min combination, which involved a 207% improvement over untreated biomass (159 mL $CH_4·g^{-1}_{VS}$).

Yang et al. (2019) analyzed the effect of different filter media: perlite (P), ceramsite (C) and rubber granules (R) on the DA efficiency of pig manure in a leaching bed reactor coupled to a continuous stirred tank reactor (LBR-CSTR). Ceramsite showed the best performance, its biogas production was 241.68 $mL·g^{-1}_{VS}$, 1.24 times higher than that of the control CSTR (CK); while for methane, a yield of 137.39 mL $CH_4·g^{-1}_{VS}$, higher than that obtained by CK (77.58 mL $CH_4·g^{-1}_{VS}$). The organic degradation was 86.82%, 19.49% higher than that of CK.

Fan et al. (2020) studied the feasibility of adding nanobubble water (NBW) to improve digestion stability, methanogenesis efficiency and related mechanisms in pig manure AD. For this purpose, they performed treatments with and without NBW addition at four TS concentrations: 3, 6, 8 and 10%. Inhibition by volatile fatty acid (VFA) accumulation occurred when TS was 8% without NBW addition. Methane yield was 201-230 mL $CH_4·g^{-1}_{VS}$ in the reactors with NBW at TS of 3-6%, approximately 20.3-25.0% higher than the control reactors. In addition, higher water mobility and zeta potential in NBWs were found to promote soluble protein and carbohydrate consumption during the AD process.

Wang et al. (2021) added biochar at concentrations of 3, 5 and 7% dry base to investigate its influence on biogas production and reduction of heavy metal bioavailability during AD of pig manure. Methane yield increased 26.7%, 23% and 26.4% after the addition of 3%, 5% and 7% biochar, with respect to the control. Additionally, with the addition of 5% biochar, the highest passivation rate of Ni, As and Pb was shown, while the highest passivation rate of Cd, Cr, Mn and Zn was observed with 7% biochar.

For co-digestion, Dechrugsa et al. (2013) evaluated the effects of inoculum to substrate ratio (ISR) and substrate mix ratio on co-digestion of grass and pig manure using different inoculums: one from a rubber latex factory (RLD) and other from pig waste slurry (PFD). Co-digestion was performed at five different substrate ratios (G): 0:100, 25:75, 50:50, 75:25 and 100:0, and four inoculum-substrate levels (ISR): 1, 2, 3, and 4. Each of the ISRs was achieved by maintaining a constant inoculum concentration of 20 $g_{VS} \cdot L^{-1}$ and varying the substrate concentration in the range of 5-20 $g_{VS} \cdot L^{-1}$. RLD showed higher methanogenic activity (41.4 mL $CH_4 \cdot g^{-1}_{VS}$) than PFD (37.3 mL $CH_4 \cdot g^{-1}_{VS}$). In contrast, the maximum methane yields produced with the highest ratio of grass mixture were 369.6, 437.6, 465.9, and 442.6 mL $CH_4 \cdot g^{-1}_{VS}$ for RLD, as compared to 332.4, 475.0, 519.5 y 521.9 mL $CH_4 \cdot g^{-1}_{VS}$ for PFD inoculum at ISR 1, 2, 3, and 4, respectively.

Astals et al. (2015) carried out a co-digestion with *Scenedesmus* sp. with and without extraction of intracellular algal co-products by pretreatments with free nitrous acid and solvent Soxhlet extraction, respectively. Pig manure was co-digested in three substrate ratios with the crude algae (85:15, 70:30 and 50:50) and two substrate ratios pig manure-each algal residue (85:15% and 70:30%). In addition, BMP assays of each individual substrate and the three algal residues were conducted. Pretreatment increased the methane yield of algae by 29% to 37% compared to raw algae; however, co-digestion showed a synergy among pig manure and raw algae that increased the methane yield of raw algae from 163 to 245 mL $CH_4 \cdot g^{-1}_{VS}$ with a substrate ratio of 85:15, which was not the case with the pretreated algae-pig manure co-digestion.

Wang et al. (2016) realized a co-digestion with *Chlorella* sp. (A). Six different PM:A ratios were had: 100:0, 94:6, 84:16, 57:43. 25:75 and 0:100 VS-basis. The best yield was obtained with the 94-PM:6-A treatment (348 mL $CH_4 \cdot g^{-1}{}_{VS}$), followed by that obtained with solo PM (317 mL $CH_4 \cdot g^{-1}{}_{VS}$) and 84-PM:16-A treatment (311 mL $CH_4 \cdot g^{-1}{}_{VS}$), subsequently, the yield decreased as the A content increased (292-250 mL $CH_4 \cdot g^{-1}{}_{VS}$).

Cárdenas-Cleves et al. (2018) employed FW as co-substrate at different FW:PM ratios: 100:0, 80:20, 60:40, 50:50, 40:60, 20:80 and 0:100. Additionally, they compared the BMP of these co-digestions with (WN) and without nutrients (NN) added. The highest methane yields were obtained for the FW:PM 60:40 WN and NN ratio with values of 72.87 and 62.83 mL $CH_4 \cdot g^{-1}{}_{VS}$, respectively; representing an increase of 27 (WN) and 13% (NN) compared to FW mono-digestion.

Finally, Abudi et al. (2022) used mango leaves (ML) with five different ratios of ML:PM (1:0, 3:1, 1:1,1:1,1:3, and 0:1) VS-basis as co-substrate. The methane yields obtained showed that the addition of PM greatly improved the methane production of ML. The highest biodegradability was 86% for the 1:3 treatment; improved by 19-160% over the rest of the treatments. Methane yield (465 mL $CH_4 \cdot g^{-1}{}_{VS}$), also for the 1:3 treatment, was 196%, 37%, 24% and 66% higher than the 1:0, 3:1, 1:1 and 0:1 treatment, respectively.

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3. SARGASSUM-PIG MANURE CO-DIGESTION SYNERGISTIC EFFECT ON BIOCHEMICAL METHANE POTENTIAL

3.1 Introduction

The excessive deposition of sargassum in the shores all around the world has caused several problems in the last decade. Different sargassum species (S) have been identified as invasive macroalgae, each one of them present in certain geographical zones, such as *S. horneri* and *S. muticum* have been reported in Japan and Korea; *S. horneri* in Taiwan, Morocco, and Argel. *S. polycistum* was found in China, which later spread to Oceania; *S. muticum* has been found in Europe (Devault et al., 2021).

To dimension the problem, Wang and Hu (2016), used the satellite images from 2000 to 2015, to study both the distribution and coverage of sargassum in the Central West Atlantic. These authors found that the annual mean and summer mean coverages during the year of 2015 were four times bigger than the ones in 2011. In particular, summer mean coverage during 2015 was 20 times bigger when it was compared to the years 2009 and 2010. The sargassum produced in the Northwest Gulf of Mexico exports yearly one million tons of wet sargassum to the Atlantic (Gower and King, 2011). In 2018, the coverage area in the Atlantic was almost 3,000 km^2, registering a biomass of more than 20 million tons (Wang et al., 2019). In the Mexican Caribbean, the massive influx started at the end of 2014, with peaks in 2015, 2018, and 2019, having a coverage range from 6,500 to 22,900 ha. The average annual waste recollection was 3,200 and 1,700 m^3/km/month for 2018 and 2019, respectively (Chávez et al., 2020). Based on previous studies, the negative impact on the tourism economy is attributed to the production of hydrogen sulfide during the decomposition of these macroalgae (Yuhendra et al., 2021), resulting in unpleasant odors and detrimental effects on human health. In addition, accumulation of sargassum is visually annoying, and as a result, the economy of the region was affected due to downfall in tourists arrival.

Coinciding this, a reduction in hotel occupancy rates in Cancun and Riviera Maya was observed after 2018 (2.87% in the last months of the year to April 2019) (Vinagre et al., 2019). The Mexican government invested US$17 million in the removal of 522,226 tons of sargassum in 2018 and US$2.6 million for 85,000 tons in 2019 (Espinosa and Ng, 2020), while the hotel sector spent between US$128,770 and US$284,830 in 2018 on staff salaries just to clean their beaches and transport sargassum to disposal sites (Salter, 2020).

Once the sargassum has been collected, it is necessary to establish measures so that its disposal does not trigger more adverse effects than those already mentioned. Thus, anaerobic digestion could be an alternative for bioenergy production from Sargassum, due to its chemical composition. Methane yields ranging from 60 to 541 mL CH_4, from species such as *S. muticum, S. fluitans, S. natans, S. polycystum and S. fulvellum* have been reported. It should be noted that most of these works are on mono-digestion of sargassum biomass. One of the strategies followed by the authors was to apply pretreatments to sargassum to reduce the inhibitory factors and improve the yields. For example, Costa et al. (2015) applied heat treatment in autoclave, 121 °C, 1 bar for 15 min, and reported a maximum yield of 541 mL $CH_4 \cdot g^{-1}_{VS}$ using 2.5 g_{VS} and 0.09 $g \cdot L^{-1}$ of inoculum. Milledge and Harvey (2016) pre-treated biomass by ensiling, and obtained as maximum yield of 110 mL $CH_4 \cdot g^{-1}_{VS}$. Tapia-Tussell et al. (2018) biologically treated sargassum biomass with *Trametes hirsuta*, a laccase producing fungus, which resulted in a final production of 104 mL $CH_4 \cdot g^{-1}_{VS}$. Farghali et al. (2021) employed cellulase treatment, which yielded 186.60 mL $CH_4 \cdot g^{-1}_{VS}$. Flórez-Fernández et al. (2021) treated the biomass with pressure and autohydrolysis treatments; 150 °C, and obtained a yield of 170 mL $CH_4 \cdot g^{-1}_{VS}$. Maneein et al. (2021) carried out multiple washes and compared the yield of sargassum collected in spring and summer, and reported that the highest methane production was from sargassum collected in summer (170.7 mL $CH_4 \cdot g^{-1}_{VS}$). Finally, Abomohra et al. (2021) compared a pre-fermentation, and concluded that the best yield was with crude biomass (172.7 mL $CH_4 \cdot g^{-1}{}_{VS}$). There are also some studies related to other sargassum species (see **Supplementary material**).

There are several reports on the positive impacts of co-digestion, viz, improving the yield from substrates having wide C:N ratio. Espinosa-Solares et al. (2022) reported between 80 and 90% increase in methane yield from a co-digestion cow manure and cactus cladode, compared to the mono-digestion systems yield. Abudi et al. (2022) reported that methane yield from pig manure and mango leaves co-digestion increased by 66 to 196%, over the mono-digestion of either substrate. Thompson et al. (2021) reported that co-digestion of sargassum (**S**) and food waste (**FW**), yielded a maximum value of 292.18 mL $CH_4 \cdot g^{-1}{}_{VS}$, at a ratio of 1:3 with a C:N ratio of 17:1 and pressure and hydrothermal pretreatments were used in this study.

The co-digestion systems for sargassum will depend on the particular residuals available in the area where the macro-algae are collected. For example, in the Mexican Caribbean shore, there are two main species as follows: *S. natans* and *S. fluitans*. The Yucatan Peninsula in Mexico, in addition to being affected by the accumulation of sargassum, also has an excessive production of waste derived from pork industry due to the growing demand. In the period from 2010-2019, pork production showed an annual increase of 6.5%, consequently, producers implemented intensive breeding systems to meet the demand, which resulted in high accumulation of pig manure (FIRA, 2020).

With respect to pig manure (**PM**), co-digestion studies have been carried out with grass (Dechrugsa et al., 2013), *Scenedesmus sp*. (Astals et al., 2015), *Chlorella sp.* (Wang et al*.,* 2016), FW (Cárdenas-Cleves et al*.,* 2018), and mango leaves (Abundi et al*.,* 2022), with yields ranging from 72 to 655 mL $CH_4 \cdot g^{-1}{}_{VS}$. There are also some studies of methane production from pig manure in mono and co-digestion (see **Supplementary material**).

Considering that there are still no reports of sargassum-pig-manure co-digestion and in virtue of incorporating these two wastes that represent an important ecological impact in the Yucatan Peninsula, the objective of this study was to assess the Biochemical Methane Potential (BMP) using sargassum in co-digestion with pig manure.

3.2 Material and methods

3.2.1 Experimental procedure

Feed was prepared using sargassum (S) (*Sargassum* spp.) and pig manure (PM), having a target concentration of 3.0% of total solids (TS). Sargassum was collected from Cancun, Quintana Roo, Mexico (21°03'17.0"N 86°46'49.6"W) and pig manure was obtained from an experimental farm located in Chapingo, Estado de Mexico, Mexico. Different ratios of S:PM, were tested: 0:100 (0S-100PM), 30:70 (30S-70PM), 50:50 (50S-50PM), 65:35 (65S-35PM) and 100:0 (100S-0PM). Inoculum was taken from an active biodigester, which has been fed for five years with chicken litter at 3% of TS and enriched with sodium propionate at 30 $g \cdot L^{-1}$. Serum bottles, 305 mL, with an initial working volume of 230 mL were used as the microcosms. Substrates were dried and milled previous to slurry preparation. Anoxic water was used to achieve a 3.0% TS for each treatment, inoculated (10%; v/v), and hermetically sealed with a rubber septum and metal cap and were purged using nitrogen gas to displace oxygen. Incubation was done at 37 ± 1 °C for 100 days. The chemical composition of the different treatments is shown in **Table 1.**

3.2.2 Analytical methods

The composition of the substrates was carried out using elemental analyzer (Perkin Elmer PE2400) and is presented in **Table 2.** Determination of heavy metals and other elements was carried out with an atomic absorption spectrophotometer AIN-E-025, an AIN-E-010 flame photometer, an Ultraviolet-visible spectrophotometer AIN-E-011 and an Optical Emission Spectrophotometer Coupled to Plasma AIN-E-004. Total solids (TS), fixed solids (FS) and volatile solids (VS), alkalinity and chemical oxygen demand (COD) were determined at the beginning and end of the experiment by following the standard APHA methods (1998), and the pH was quantified with a potentiometer (Conductronic, PH140).

The volatile fatty acid (VFA) profile was tested weekly for acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, isocaproate, caproate and heptanoate. Two mL of effluent were taken from each microcosm and were centrifuged (MiniSpin Plus micro-centrifuge Eppendorf, Germany) for 10 minutes at 14500 rpm. The supernatant acidified to pH 3.0 with HCl, it was centrifuged again and then filtered. Five μL were injected into a gas chromatograph (Claurus 500, Perkin Elmer, USA) equipped with a flame ionization detector (FID), under the following operating conditions: Helium was used as the carried gas with a gas

Table 1. Initial physical and chemical composition of the treatments

Treat.	Substrates		C:N	TS	VS/TS	pH	COD	Alkalinity
	S	PM		[% D.B.]	[%]		[mg·L^{-1}]	[mg·L^{-1}]
1	**0**	**100**	11.0	3.10 ± 0.1[a]	61.63 ± 5.8[a]	8.07 [a]	32,630.56 ± 1,383.04[a]	3,102.08 ± 173.72[b]
2	**30**	**70**	14.5	3.00 ± 0.0[a]	58.06 ± 2.5[a]	7.95 [a]	21,491.67 ± 1,010.36[b]	3,846.58 ± 108.17[a]
3	**50**	**50**	16.8	3.14 ± 0.1[a]	38.56 ± 3.4[b]	8.15 [a]	18,352.78 ± 1,457.20[bc]	2,298.37 ± 131.32[b]
4	**65**	**35**	18.5	2.97 ± 0.0[a]	29.65 ± 1.4[cb]	8.17 [a]	15,825.00 ± 1,087.59[c]	2,729.83 ± 150.95[b]
5	**100**	**0**	22.6	3.02 ± 0.1[a]	14.47 ± 1.6[c]	8.21 [a]	15,575.00 ± 463.98[c]	2,804.28 ± 108.17[b]

Means with the same letter in the same column do not present significant statistical difference, Tukey ($p<0.05$)

Table 2. Elemental analysis of substrates

Substrates	% TS	% FS	% VS	% C	% H	% N	% S
Sargassum	89.80	49.20	50.70	27.10	3.80	1.20	0.90
Pig manure	27.20	19.10	80.80	37.50	5.70	3.40	0.70

flow of 5 mL·min^{-1} at 10.6 psi, 180 °C injection port, oven temperature was programed from 100 °C for 8 min with ramp of 160 °C for the next 8 min, 250 °C as detector temperature. The concentration of VFAs were calculated using a calibration curve obtained from Free Volatile Acids Mix of analytical grade (CRM-46975, Sigma-Aldrich, USA) as standard, under the same operating conditions.

Biogas production was quantified by the displacement method in saline water using 10% NaCl. The methane percentage was obtained by injecting 10 μL of biogas from each microcosm into a gas chromatograph (Claurus 500, Perkin Elmer, USA) with the following operating conditions: gas flow of 14 mL·min^{-1} at 14.0 psi, 100 °C injector port, 70 °C oven and 150 °C detector with a retention time of 4 min with helium as carrier gas. The percentage of methane was obtained using a calibration curve with pure methane (HDSP No. P-4618-F, Praxair®, Mexico) as standard. Methane produced is reported at normal temperature and pressure conditions (20 °C and 585 mmHg respectively). Biogas and methane concentration were monitored daily.

3.2.3 Modeling and statistical analysis

The methane production results were adjusted to the modified Gompertz model (MGM) (Zwietering et al., 1990) which is presented in **Eq(1)**:

$$AMY = BMP \cdot exp\left\{-exp\left[\frac{\mu_m \cdot e}{BMP}(\lambda - t) + 1\right]\right\} \qquad (1)$$

AMY is the accumulated methane yield (mL $CH_4 \cdot g^{-1}{}_{VS}$) at time t, BMP is the biochemical methane potential (mL $CH_4 \cdot g^{-1}{}_{VS}$) μ_m is the rate of methane production per day (mL N $CH_4 \cdot g^{-1}{}_{VS}$ d^{-1}), e is the mathematical constant (2.718282), λ is the time of the phase lag (d), t is the digestion time (d). The model was adjusted using SigmaPlot version 13. The parameters obtained with the MGM (BMP, μ_m and λ) were evaluated by ANOVA and a further comparison of means was performed by Tukey test (SAS System 9.0) in order to determine significant statistical differences among treatments. Principal Component Analysis was performed using SAS System (9.0).

3.3 Results and discussion

3.3.1 Methane Production

The VFA concentration and AMY as a function of time for each treatment are shown in **Figure 1.** The highest accumulated yield was obtained with 50S-50PM (441.1 mL $CH_4 \cdot g^{-1}_{VS}$), followed by the 65S-35PM treatment (415.6 mL $CH_4 \cdot g^{-1}_{VS}$) and the 30S-70PM treatment (319.6 mL $CH_4 \cdot g^{-1}_{VS}$). It can be observed that methane production at 50S-50PM treatment recorded a 138.7% increased in the yield in comparison to the mono-digestion of 100S-0PM (318.8 mL $CH_4 \cdot g^{-1}_{VS}$) and by 1,336.4% with respect to the 0S-100PM mono-digestion (33.0 mL $CH_4 \cdot g^{-1}_{VS}$). These results confirm the synergistic effect in the co-digestion treatments.

The concentrations of COD, TS and VS, as well as pH and alkalinity were quantified after 100 days of digestion and are presented in **Table 3**. Percentage of COD removal ranged from 20.94 to 41.32%. A similar behavior was observed in the case of VS_{Fed} degradation, which was in the range of 30.56 to 45.53%. The highest degradation values were observed in the treatment recording the highest BMP.

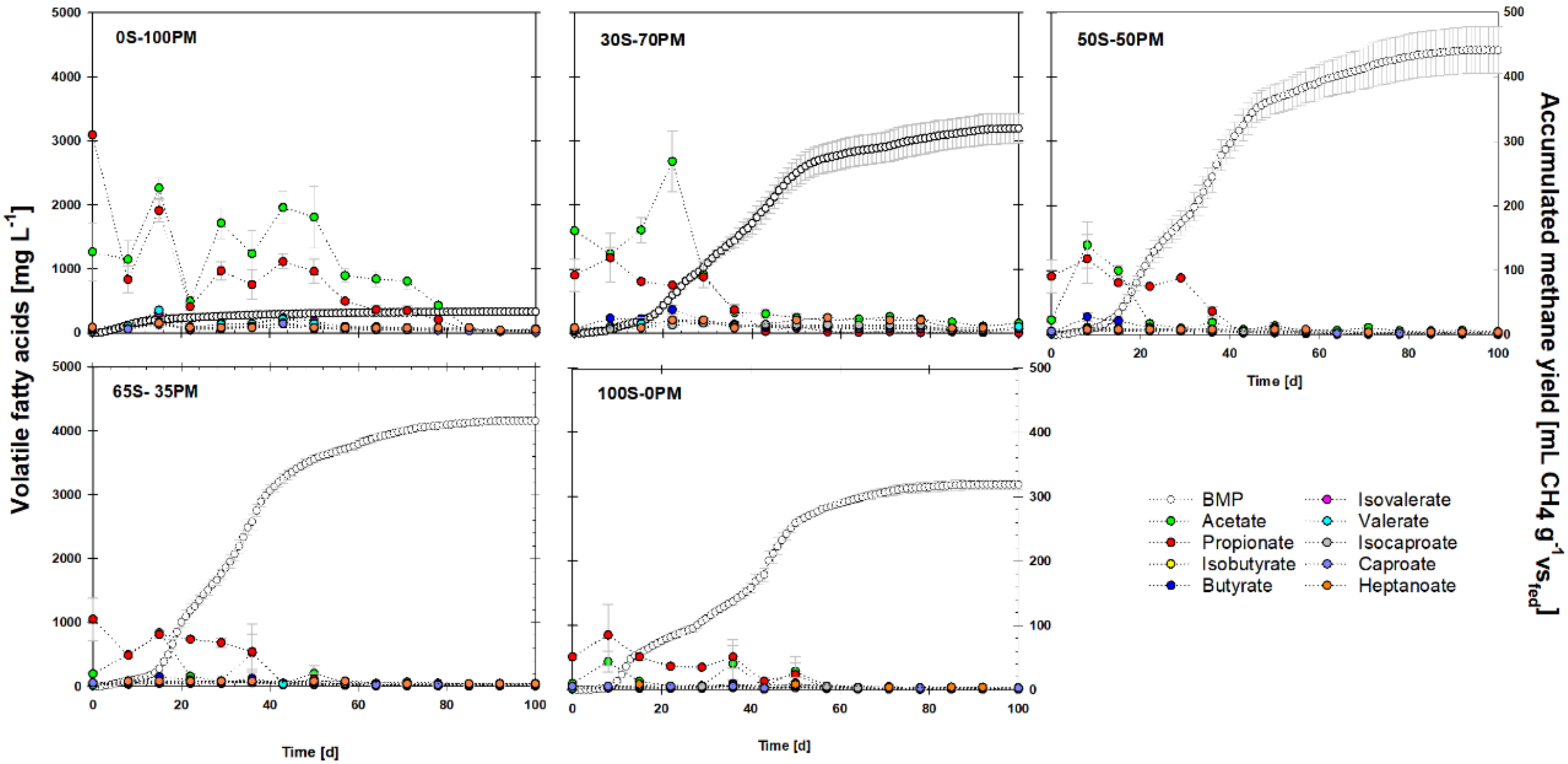

Figure 1. Accumulated methane yield and VFA profile during assays.

Table 3. Final physical and chemical composition of the treatments

Treat.	Substrates		TS	VS/TS	pH	COD	Alkalinity
	S	**PM**	**[% D.B.]**	**[%]**		**[mg·L^{-1}]**	**[mg·L^{-1}]**
1	**0**	**100**	2.06 ± 0.00[ab]	52.22± 5.71[a]	6.55 [b]	25,797.22 ± 1,128.68[a]	12,740.73 ± 475.75[a]
2	**30**	**70**	2.32 ± 0.26[ab]	51.79± 1.74[a]	8.23 [a]	14,436.11 ± 1,564.46[b]	7,990.97 ± 666.06[b]
3	**50**	**50**	2.58± 0.16[a]	46.78 ± 4.33[a]	8.20 [a]	10,769.44 ± 1,873.51[b]	5,947.73 ± 133.13[c]
4	**65**	**35**	1.71 ± 0.10[b]	39.31 ± 1.45[a]	8.32 [a]	10,075.00 ± 506.90[b]	5,004.69 ± 179.15[dc]
5	**100**	**0**	2.16 ± 0.22[ab]	10.44 ± 1.42[b]	8.30 [a]	9,825.00 ± 333.33[b]	3,358.52 ± 372.07[d]

Means with the same letter in the same column do not present significant statistical difference, Tukey (*p<0.05*)

3.3.2 Biochemical Methane Potential

The AMY, as a function of time, was fitted to the MGM for each replication of all treatments. The result of applying ANOVA to the MGM parameters (BMP, μ_m and λ), along with the Tukey test, is presented in **Table 4**.

Table 4. Gompertz model parameters for methane production from Sargassum and or/pig manure

Substrates	BMP	μ_m	λ	R^2
0S-100PM	31.7^{c}	1.3^{c}	0.0^{c}	0.967
				0.976
				0.954
30S-70PM	322.4^{b}	7.7^{b}	15.5^{a}	0.998
				0.997
				0.995
50S-50PM	441.5^{a}	11.3^{a}	13.1^{ab}	0.997
				0.997
				0.998
65S-35PM	415.3^{ab}	11.8^{a}	13.2^{ab}	0.998
				0.999
				0.996
100S-0PM	338.7^{b}	6.7^{b}	11.9^{b}	0.977
				0.992
				0.987

Means with the same letter in the same column do not present significant statistical difference, Tukey ($p<0.05$)

It is important to note that the BMP from MGM corresponds to the maximum theoretical accumulated yield for an infinite time. The BMP comparison indicated that it varies from 31.72 to 441.47; the 50S-50PM treatment showed the highest BMP value, however, did not showed any statistical difference with 65S-35PM treatment. From the statistical point of view the second group of BMP consisted of the treatments 30S-70MP and 100S-0PM. The lowest BMP was obtained with the 0S-100PM treatment and was statistically different from all the other treatments. Regarding methane production rate, the behavior was similar to BMP profile, with the lowest value obtained for the 0S-100PM treatment (1.29 mL $CH_4 \cdot g^{-1}_{VS}$ d^{-1}) and the highest for the 65S-35PM treatment (11.84 mL $CH_4 \cdot g^{-1}_{VS}$ d^{-1}), which did not show a significant statistical difference with the 50S-50PM treatment (mL $CH_4 \cdot g^{-1}_{VS}$ d^{-1}). However, it is important to highlight that the 0S-100PM treatment practically began to produce methane immediately without any lag phase, compared to the rest of the treatments, where the lag phase ranged from 11 to 15 days.

3.3.3 Effect of C:N on BMP

The influence of the C:N ratio on the BMP for the treatments in comparison to the available literature data is presented in **Figure 2a**. It can be observed that the C:N ratio of the different treatment was in the range of 11.0 to 22.6, with the 0S-100PM treatment showing the lowest value and with the 100S-0PM treatment recorded the highest value. The highest BMP recording 50S-50PM treatment showed a C:N ratio of 16.8. It can be seen from **Figure 2a** that all of them followed the same trend. Milledge et al. (2020), evaluated the BMP at C:N ratio ranging from 17.4 to 22.1, and reported the maximum BMP value at 17.4 C:N ratio. Thompson et al. (2021) tested different ratios of co-digestion of sargassum and food waste, with and without hydrothermal pretreatment and the maximum reported value ranged from 16.3 to 18.8. The results of the present study and studies realized by Milledge et al. (2020) and Thompson et al. (2021) demonstrated that the relative optimum C:N ratio values for *S. fluitans* and *S. natans* ranged from 16.3 to 18.8.

In order to compare the data, a relative BMP was obtained for each data set, and expressed as the percentage of the maximum value for the specific data set. **Figure 2b**, shows that the relative BMP have the same trend for all data in **Figure 2a**, having similar slopes and the maximum values are relatively close to each other.

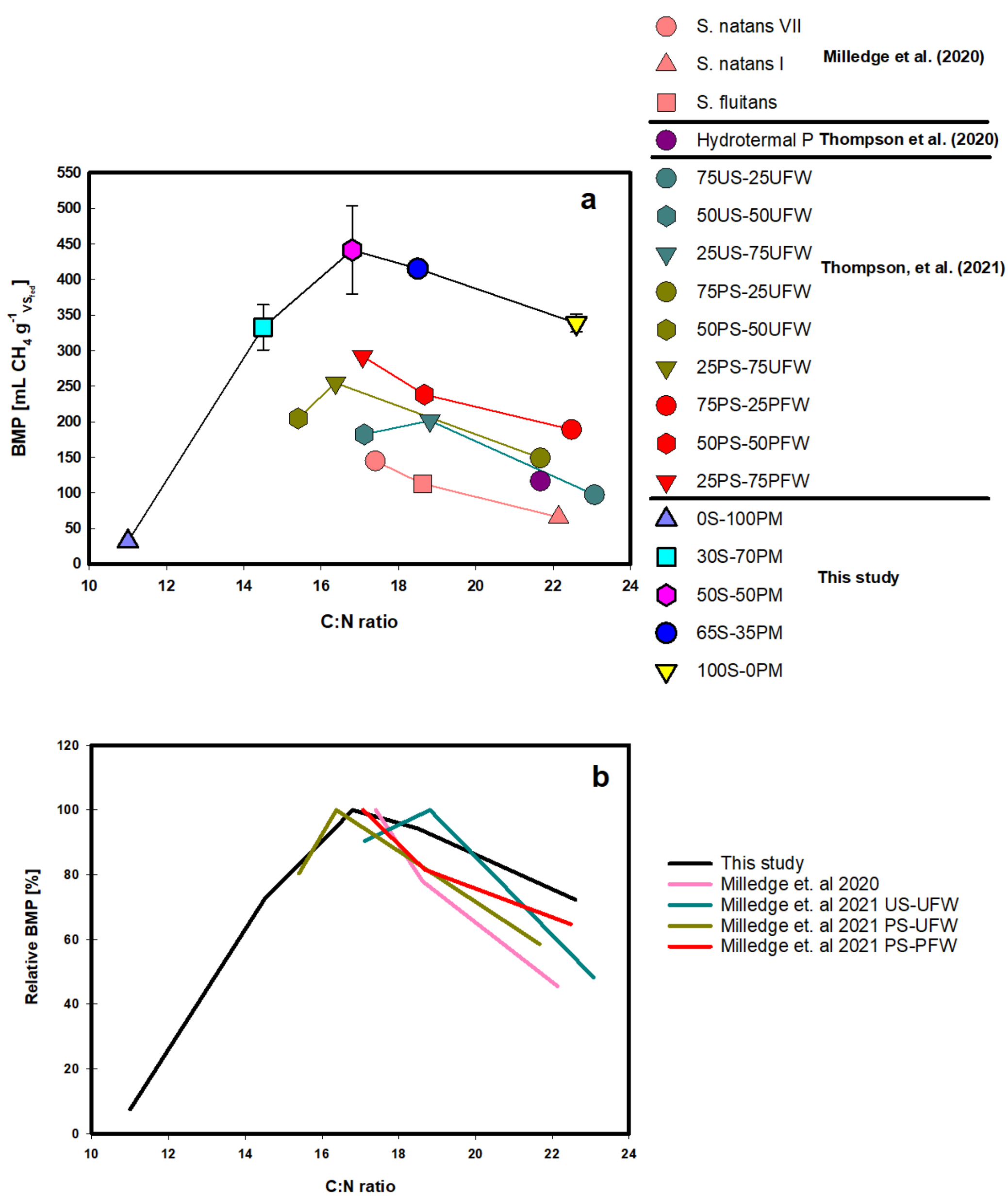

Figure 2. Influence of the C:N on BMP for treatments and literature reports.

3.3.4 Kinetics of VFA profile

Three most abundant VFA formed during the progress of anaerobic digestion, i.e., acetate, propionate, and butyrate concentrations were taken and was analyzed by PCA. It was observed that three components explained 93.3% of the variability. **Figure 3** shows the distribution of the treatments using the main three components. It is clearly seen that the 65S-35PM and 100S-0PM treatments are located together. Based on that information, the acetate, propionate, and butyrate were plotted for the four different behaviors and presented in **Figure 4** and **Figure 5.**

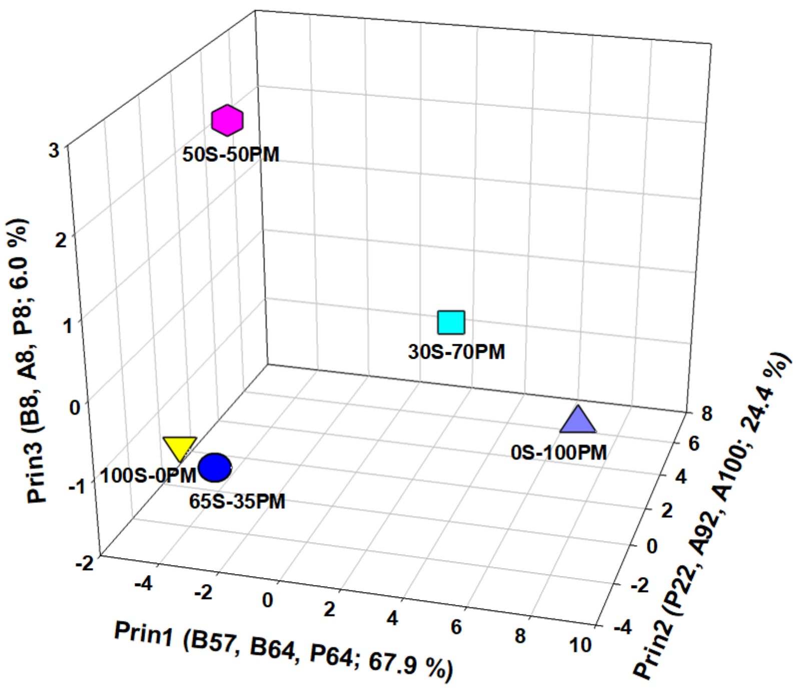

Figure 3. Principal Component Analysis based on VFAs profiles.

- **Solo pig manure (0S-100PM):** In this case, there was an accumulation of acetate, propionate, and butyrate (**Figure 4. a, b, c**). This treatment showed the highest concentrations of the VFA and their consumption was slower. Based on the observed high acetate concentration, there could be inhibition of acetotrophic methanogenic activity. On the other hand, an inhibition of propionate oxidation or a change in acidogenesis could also

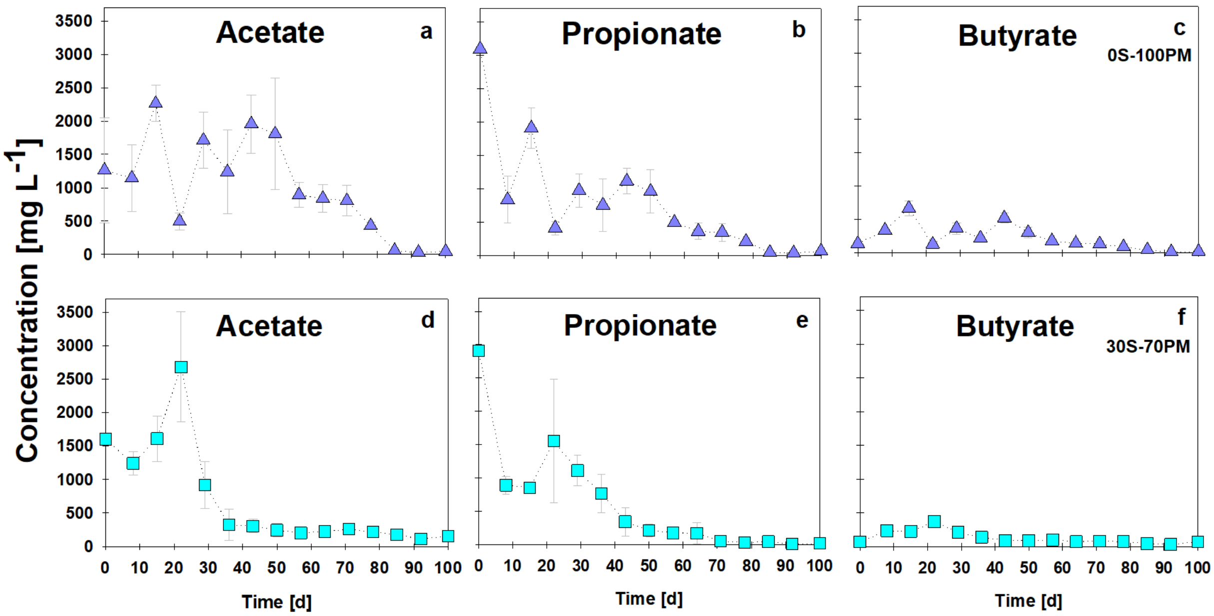

Figure 4. Changes on main VFAs profiles during assays for 0S-100PM and 30S-70PM treatments.

have occurred, given that increased partial pressure of hydrogen modify the pathway towards longer VFA production than acetate (Polizzi *et al*. 2018). One another possible reason could be attributed to heavy metal concentration (**Table 5**). Chen et al. (2008) mentioned that high concentrations of Cu cause inhibition of methanogenesis due to H_2 production and VFA accumulation. Lin (1992) reported that the LC_{50} of Cu and Zn was 12.5 and 16 mg kg^{-1}, respectively, for acetotrophic methanogens. On the other hand, Zayed and Winter (2000) reported that Cu and Zn caused 50% inhibition of mixed methanogens at 10 and 40 mg kg^{-1} respectively. Karri et al. (2006) reported that LC_{50} of Cu was 20.7 mg kg^{-1} and 8.9 for acetotrophic and hydrogenotrophic methanogens respectively. It can be see from the results of this study that Cu (22.97 mg kg^{-1}) and Zn (44.63 mg kg^{-1}) concentrations were higher than the values reported and could have affected the methane yield obtained in this study.

Some authors also have reported inhibition due to the presence of antibiotics or other drugs in animal manure; however, this was not the case

Table 5. Heavy metals and other elements in feed

Element	0S-100PM	30S-70PM	50S-50PM	65S-35PM	100S-0PM
Potassium mg kg^{-1}	553.50	578.23	594.72	607.09	635.94
Calcium mg kg^{-1}	486.50	1,588.81	2,323.69	2,874.85	4,160.88
Magnesium mg kg^{-1}	310.22	541.41	695.54	811.14	1,080.86
Sodium mg kg^{-1}	483.00	579.03	643.05	691.07	803.10
Copper mg kg^{-1}	22.97	17.28	13.49	10.65	4.02
Iron mg kg^{-1}	81.57	59.08	44.09	32.85	6.61
Manganese mg kg^{-1}	15.45	11.31	8.56	6.49	1.67
Zinc mg kg^{-1}	44.63	31.36	22.52	15.89	0.41
Molybdenum mg kg^{-1}	0.18	0.17	0.17	0.16	0.15
Selenium mg kg^{-1}	0.50	1.31	1.85	2.26	3.21
Cobalt mg kg^{-1}	0.02	0.04	0.06	0.07	0.10
Lead mg kg^{-1}	0.22	0.16	0.11	0.08	0.01
Chromium mg kg^{-1}	0.81	0.59	0.45	0.34	0.09
Nickel mg kg^{-1}	1.16	0.89	0.71	0.58	0.27
Arsenic mg kg^{-1}	0.13	0.76	1.18	1.50	2.24
Cadmium mg kg^{-1}	0.04	0.03	0.02	0.01	ND

ND: not detected by the method used

Based on TS

in this work, due to the fact that the only compound fed to the pigs at the time of manure collection was ractopamine at a concentration of 10 ppm. According to the work of Dos Santos et al. (2016), this compound did not affect biogas production, but on the contrary, there was a higher biogas production with a concentration of 20 ppm when pig manure was supplemented for 28 days. Another drug that could have affected methanogenesis is Ivermectin, which was fed to the pigs three months prior to collection as a deworming agent. There are no papers reporting the influence of ivermectin on anaerobic digestion; however, Halling-Sorensen et al*.* (1998) reported that the duration of after-effect ivermectin treatment depended on species, temperature and type of livestock. Halley et al. (1993) observed that macrocyclic lactones, a group to which this deworming agent belongs, are susceptible to aerobic biodegradation in soils; although, ivermectin seems to be quite persistent in manure under appropriate conditions (Sommer and Steffansen, 1993). Hence it is unlikely that an inhibitory effect of this compound has occurred.

Is well known that the accumulation of VFA can lead to acidification of the medium and in this manner the AD process inhibition (McCarty and Smith 1986), in the case of this treatment, the pH dropped to 6.5, and can be related to the low BMP value. The accumulation of VFA occur due to a metabolic imbalance between acid producers and consumers, or through direct inhibition of the latter (Mathai et al*.,* 2020).

- **30S-70PM:** This treatment shows its highest accumulation of acetate on day 22, followed by a stable consumption that is maintained until the end of the experiment (**Figure 4. d, e, f**). This behavior can also be observed in the case of propionate and butyrate. It is possible to see that, in spite of their initial accumulation, there was an almost immediate consumption of the three fatty acids, which is the opposite case of the treatment with solo pig manure. It is important to highlight that it is the treatment that exhibits the highest final concentrations of acetate and butyrate (154.94 mg L^{-1} and 71.68 mg L^{-1} respectively), so a possible inhibitory effect related to the higher amount of PM present in the treatment can still be considered
- **50S-50PM:** Acetate exhibited its highest peak on day 7, and the consumption was observed from day 15 (**Figure 5. g, h, i**). A similar behavior was observed with butyrate, but not with propionate, which again showed an accumulation around day 29 and later proceeded to its consumption. It should be noted that there is no significant accumulation of VFA at the beginning, but rather it takes place during the lag phase and is subsequently consumed as it is produced. It is the treatment with the

highest percentage of propionate consumption (99.3%), which may be related to a better adaptation of the inoculum, accustomed to a medium with high propionate concentrations, in addition to the synergy between the substrates.

- **65S-35PM y 100S-0PM:** These treatments presented the lowest values for VFA accumulation; this could be related to the fact that both treatments contain a higher proportion of sargassum (**Figure 5. j, k, l**). The percentage of removal for these treatments with respect to propionate is between 98-99%, similar to the 50S-50PM treatment, however, the final AMY is lower due to lower amounts of VFA. In general, marine biomass produced low amounts of VFA. Wahab et al. (2021) reported a production of 9% acetate for *Sargassum* and lower than 2% in *Gracilaria* and *Ulva*.

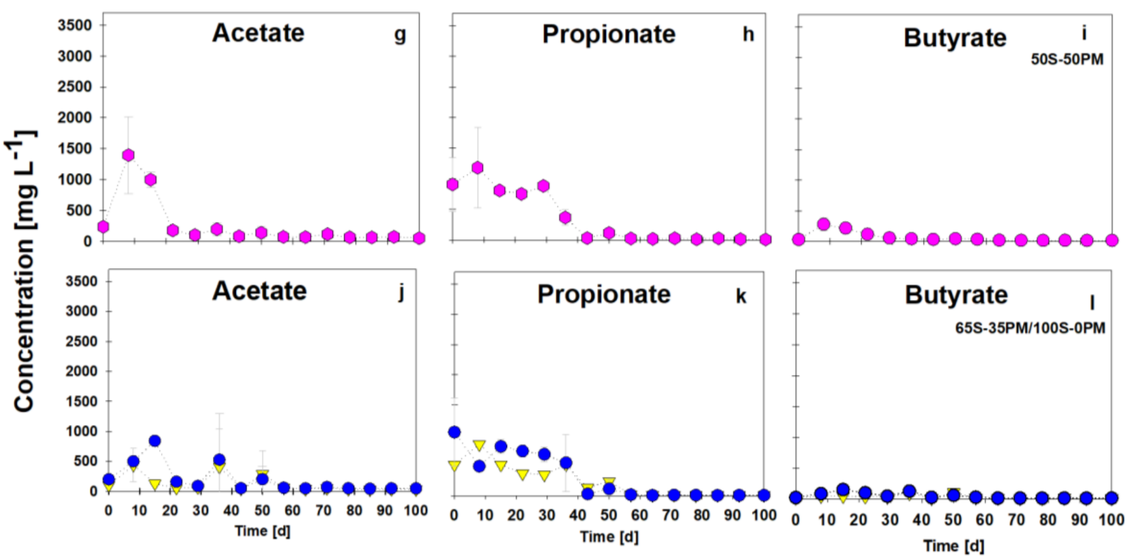

Figure 5. Changes on main VFAs profiles during assays for 50S-50PM, 65S-35PM and 100S-0PM treatments

3.3.5 Co-digestion synergistic effect

The results of this co-digestions study showed a considerable synergistic effect. Abudi et al. (2022) suggested a method for calculating the simulated BMP for a co-digestion, i.e., using a weighted average of mono-digestion yields. Thus, the synergistic effect in terms of simulated BMP (**Table 6**), for the treatments is as follows: 123.83 mL $CH_4 \cdot g^{-1}_{VS}$ for the 30S-70PM, 185.24 mL $CH_4 \cdot g^{-1}_{VS}$ for the 50S-50PM and 231.30.184 mL $CH_4 \cdot g^{-1}_{VS}$ for the 65S-35PM treatment. When these data are compared to the actual values, the synergetic effect on BMP is evident. The corresponding enhancement for the treatments, expressed as percentage of the simulated BMP, is respectively 160.4 %, 138.3 %, and 79.5 %. However, microbial diversity and their functional activity through metagenomics can explain these behaviors, in particular.

Table 6. Evaluation of co-digestion synergistic effect

Mass fraction [%]		Methane fraction [mL CH_4 g^{-1}_{VSfed}]		BMP [mL CH_4 g^{-1}_{VSfed}]		Enhancing [%]
S	**PM**	**S**	**PM**	**Simulated**	**Experimental**	
30	**70**	101.6	22.2	123.8	319.6	160.4
50	**50**	169.4	15.9	185.2	441.1	138.3
65	**35**	220.2	11.1	231.3	415.6	79.5

3.4 Conclusions

There were considerable differences in the VFA profiles of different treatments, which suggested changes in the corresponding metabolic pathways. BMP of co-digestion treatments presented higher values than mono-digestion. The synergistic effect of co-digestion in terms of BMP is clearing seen, resulting in an increase of 79.5 to 160.4% with respect to mono-digestion. The maximum BMP was 441.47 mL $CH_4 \cdot g^{-1}_{VS}$, was obtained in 50S-50PM treatment with a C:N ratio of 16.8.

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