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PRODUCCIÓN DE METANO DE ESTIÉRCOL DE CERDO EN CODIGESTIÓN CON SARGAZO Y USO DE NANOPARTÍCULAS (METHANE PRODUCTION FROM PIG MANURE IN CO-DIGESTION WITH SARGASSUM AND USE OF NANOPARTICLES)

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Para obtener el grado de:

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PRODUCCIÓN DE METANO DE ESTIÉRCOL DE CERDO EN CODIGESTIÓN CON SARGAZO Y USO DE NANOPARTÍCULAS (METHANE PRODUCTION FROM PIG MANURE IN CO-DIGESTION WITH SARGASSUM AND USE OF NANOPARTICLES)

Tesis de la **ING. JENIFFER CATHERINE GALINDO BERDUGO** bajo la supervisión del Comité Asesor indicado, aprobada por el mismo y aceptada como requisito parcial para obtener el grado de:

MAESTRA EN CIENCIA Y TECNOLOGÍA AGROALIMENTARIA

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DEDICATION

A human heart makes plans. God gives the answer.
A person's own acts seem right to the doer, but God is the weigher of souls.
Commend what you do to God, and what you plan will be achieved.
Proverbs 16:1-3

To God who has been my source of strength, support, and wisdom throughout my life.

To beloved people no longer of this world, my father Gabriel Galindo, my uncle Ricardo Berdugo, his wife Glasadis Carmona and all those family and friend's members who have passed away.

To my family, especially my mother, Dora Berdugo, and my grandparents, Rosa and Cristobal, for giving me the best gift I ever had. The love, kindness, and generosity of your heart have inspired me to strive to be as resilient as you have been.

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RESUMEN GENERAL

PRODUCCIÓN DE METANO DE ESTIÉRCOL DE CERDO EN CODIGESTIÓN CON SARGAZO Y USO DE NANOPARTÍCULAS

La Península de Yucatán es el sumidero de carbono más importante de México y el reservorio de agua subterránea más importante del mundo. Sin embargo, la eliminación inadecuada del estiércol de las granjas porcinas industriales y la llegada masiva de *Sargassum* spp. a las costas amenazan estos servicios ecosistémicos. Mediante la digestión anaerobia (DA), estos residuos pueden convertirse en energía renovable. A pesar de ello, la producción de biogás a partir de biomasa algal produce bajos rendimientos de metano. Para mejorar la eficiencia del sistema de digestión anaerobia, se ha considerado útil la adición de materiales conductores como las nanopartículas de magnetita. Por lo cual este estudio investigó la codigestión anaerobia de sargazo (S) y estiércol de cerdo (PM). Así como la adición de 20 $mg{\cdot}L^{-1}$ de nanopartículas (NPs) de magnetita (Fe_3O_4) sintetizadas por coprecipitación. Se evaluaron seis tratamientos diferentes a nivel de microcosmos (0S-100 PM, 15S-85 PM, 30S-70 PM, 50S-50 PM, 65S-35 PM, 100S:0PM) para elucidar su influencia sobre el potencial bioquímico de metano (PBM). En consecuencia, de la adición de nanopartículas de magnetita, el mayor rendimiento acumulado de metano se obtuvo con el tratamiento 30S-70PM (369.1 ± 6.9 mL $CH_4{\cdot}g_{sva}^{-1}$), seguido de 50S-50PM (365.0 ± 7.2 mL $CH_4{\cdot}\ g_{sva}^{-1}$). Demostrando que la adición de 20 $mg{\cdot}L^{-1}$ de NPs de Fe_3O_4 promovió la producción de metano en el tratamiento 30S-70PM en un 233.7% y un 85.0% en comparación con el control de sargazo y estiércol de cerdo, respectivamente.

Palabras clave: Co-digestión anaeróbica, sargazo, nanopartículas de magnetita, potencial bioquímico de metano (PBM).

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GENERAL ABSTRACT

METHANE PRODUCTION OF PIG MANURE IN CODIGESTION WITH SARGASSUM AND USE OF NANOPARTICLES.

The Yucatan Peninsula is the most important carbon sink in Mexico and groundwater reservoir in the world. However, the improper disposal of pig manure from industrial pig farms and the massive arrival of Sargassum spp. on the coasts threaten these ecosystem services. By using anaerobic digestion (AD), these wastes can be converted into renewable energy. Despite this, biogas production from algal biomass produces low methane yields. To improve the efficiency of anaerobic digestion, conductive materials such as magnetite nanoparticles have been considered useful. Therefore, this study investigated the anaerobic co-digestion of sargassum (S) and pig manure (PM). As well as the addition of 20 mg·L-1 magnetite nanoparticles (Fe3O4) synthesized by co-precipitation. Six different treatments at the microcosm level (0S-100 PM, 15S-85 PM, 30S-70 PM, 50S-50 PM, 65S-35 PM, 100S:0PM) were evaluated to elucidate their influence on biochemical methane potential (BMP). Consequently, from the addition of magnetite nanoparticles, the highest cumulative methane yield was obtained with the treatment 30S-70PM (369.1 ± 6.9 mL CH4·gvsf-1) followed by 50S-50PM (365.0 ± 7.2 mL CH4·gvsf-1). Demonstrating that the addition of 20 mg·L-1 Fe3O4 NPs promoted the production of methane on treatment 30S-70PM by 233.7% and 85.0% compared to the control of sargassum and pig manure, respectively.

Key words: Anaerobic co-digestion, sargassum, magnetite nanoparticles, biochemical methane potential (BMP).

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1. GENERAL INTRODUCTION

In Mexico, meat consumption is linked to ancestral elements, as well as to agrifood globalization (Estévez-Moreno & Miranda-de la Lama, 2022). Currently, pork is the second most consumed meat in the country, with approximately 16 kg per person per year (OECD et al., 2022). Therefore, pork farming activity is distributed throughout the national territory in different proportions, with a marked presence in Jalisco, Sonora, Puebla, and Yucatán (SIAP, 2021).

Specifically in the Yucatan Peninsula, during 2020, swine farming produced 1,819,970 head, supplying 9% of the national production. Likewise, 14.2% of the pig farms identified in the Mexican Republic are in this state, presenting an annual growth rate of 4.5%, even higher than the main pork producers in Mexico (Sonora, 2.6%, and Jalisco, 1.7%) (OCDE, 2019). In economic terms, this activity represents an evident benefit. However, environmental problems have affected the region, including groundwater contamination (Santos et al., 2022), the emission of greenhouse gases in the form of methane (CH_4) (Calvet et al., 2017), acidification, and soil eutrophication (Yuan, 2018).

The massive arrival of *Sargassum* spp. is another big waste generator. Since the summer of 2014, 2360 m^3 of sargassum per km of beach have occurred on the coasts of the Mexican Caribbean (Rodríguez-Martínez et al., 2016). According to Chávez et al. (2020), the maximum oceanic coverage of this pelagic macroalgae in the Mexican Caribbean was 22,900 ha in September 2018 and 19,000 ha in April 2019. The total amount removed from seven Mexican Caribbean beaches during 2018 and 2019 was 181,081 m^3 and 203,269 m^3, respectively (Rodríguez-Martínez et al., 2022).

The anaerobic digestion process is useful to facilitate the management of these wastes, reducing environmental impacts and obtaining potential benefits such as biogas and high-value fertilizers. However, it is a complex biological process with a low energy efficiency of 6–8% (Wei et al., 2021). Their ideal result is the production of biogas with high methane content, which is possible by developing different strategies that seek to have a more efficient process with better performance. The operating conditions, substrate type, co-digestion (Bharati & Suresh, 2017), and the addition of nanomaterials are some of them.

According to Cerrillo et al. (2021), nanoparticles (NPs) can act as electron donors or acceptors and cofactors of important enzymes to improve biogas production by strong biomass degradation through direct or indirect interspecies electron transfer (Jadhav et al., 2021). Complementarily, co-digestion of organic waste can have different advantages, including higher methane production (Zaher, 2009), better nutrient balance in the digester (Srisowmeya et al., 2021), higher utilization of organic matter (Stroot, 2001), and better system economics (Arnell, 2016).

As a result, the following hypothesis was proposed: The addition of 20 $mg \cdot L^{-1}$ Fe_3O_4 NPs will influence the anaerobic digestion performance of pig manure and sargassum. Therefore, the purpose of this research was to elucidate the influence of magnetite nanoparticles in the anaerobic co-digestion of two of the main wastes generated in the Yucatan Peninsula: *Sargassum* spp. and pig manure.

The research project was divided into three stages to achieve the stated objective. The first focused on the characterization and synthesis of magnetite nanoparticles. The synthesis of nanoparticles was carried out according to the co-precipitation method. To characterize the nanoparticles, the hydrodynamic diameter, Z-potential, and X-ray diffraction were determined. The second stage consisted of the collection and preparation of the substrates (pig manure and sargassum) to be used in anaerobic digestion. At the third stage, the microcosms were assembled in 305 mL capacity bottles for the 100-day BMP test at 37 °C± 1.

2. LITERATURE REVIEW

2.1 Anaerobic digestion

Anaerobic digestion is a complex organic compound degradation process in which the organic matter in a substrate (food waste, animal manure, and sewage sludge) is broken down in an oxygen-free environment (Xu et al., 2019). The cooperation of four diverse groups of microorganisms, such as fermentative, syntrophic, acetogenic, and methanogenic bacteria, allows the production of CH_4 and CO_2 (Demirel & Scherer, 2008).

2.1.1 Importance of anaerobic digestion

The inventory sector covering greenhouse gas (GHG) emissions and removals resulting from direct human land use, land-use change, and forestry activities (LULUCF) continued to grow throughout the period 2000-2018, reaching 14% more in 2018 than in 2000 (FAO, 2020). The inventory sector covering GHG emissions and removals resulting from direct human land use, land-use change, and forestry activities (LULUCF) continued to grow throughout the period 2000–2018, reaching 14% more in 2018 than in 2000 CO_2-eq (FAO, 2022). Hence, the agricultural challenge is to become more efficient to produce 60% food, fiber, and biofuel by 2050 to meet the increase in world population. (Pasricha et al., 2023).

To make efforts to reduce global warming at the beginning of 2021, 131 countries further committed to reduce GHG emissions to net zero by 2050. Thus, increasing the use of renewable energy instead of fossil fuels globally plays a dominant role in coping with climate change and realizing net zero emissions (Yuan et al., 2022).

The agricultural sector has enormous potential for adopting renewable sources of electricity or heat, especially using biomass waste or manure for biogas production. (UNE, 2022). Due to biogas potential to reduce GHG emissions, it ranges from soil nutrient recovery (Rizzioli et al., 2023). The capacity to replace traditional biomass fuels as firewood cooking systems (Sagastume Gutiérrez et al., 2022). The replacement of natural gas in industry processes (O'Shea et al., 2020). As well as the role it can play in meeting climate change targets under the Paris Agreement (Jain et al., 2019).

Various organic wastes, such as sewage sludge, food waste, livestock manure, and industrial wastewater, can be used as feedstock for anaerobic digestion systems (Li et al., 2021). In the process, most of the biodegradable organic matter present in the waste is converted into biogas (about 70 to 90%), which is removed from the liquid phase and leaves the reactor in a gaseous form. The main constituents of biogas are methane (CH_4) and carbon dioxide (CO_2), but there are significant quantities of undesirable contaminants such as hydrogen sulfide (H_2S), ammonia (NH_3), and siloxanes (Kadam & Panwar, 2017). Only a small portion of the organic material is converted into microbial biomass (about 5 to 15%), which then constitutes the excess sludge of the system (Lemos, 2007).

2.1.2 Anaerobic digestion process

The process of anaerobic digestion through distinct feedstock degradation phases including hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Sayara & Sánchez, 2019).

Hydrolysis

The main components of organic substrates are carbohydrates, lipids, and proteins (Sarker et al., 2019). By hydrolysis, these macromolecules are depolymerized into soluble organic monomers like sugars, long-chain fatty acids (LCFAs), and amino acids, respectively (Vavilin et al., 1996) (Arif et al., 2018). The effectiveness of this step is grounded in the presence and active action of hydrolytic and fermentative microbes (e.g., *Clostridium, Proteus Vulgaris, Bacillus, Bacteriodes, Micrococcus,* and *Staphylococcus*) to excrete extracellular enzymes (Sayara & Sánchez, 2019).

A diversity of extracellular enzymes such as amylase, cellulase, protease, and lipase, exerted by bacteria, are mandatory as catalyzers for the decomposition of each macromolecule (Sayara & Sánchez, 2019). For instance, hydrolytic enzymes (e.g., cellulase, β glucosidase, xylanase) or complex enzyme systems (e.g., cellulosome) attack polysaccharides, protease degrades proteins, and lipase is suitable for breaking down lipids (Nayono, 2014).

Hydrolysis is a rate-limiting step in biomass digestion. In the case of lignocellulosic substrates, which inhibit a specific group of cellulolytic enzymes. (Cheng & Brewer, 2021). High solid content or enzyme concentration also increases mass transfer limitations, which inhibit the process (Baksi et al., 2023). Additionally, temperature, reaction time, particle size of the substrate, and pH would affect the hydrolysis efficiency (Lin et al., 2012).

Considering that its performance may directly influence the subsequent processes of anaerobic digestion, a great deal of attention has been focused on improving hydrolysis kinetics (Meegoda et al., 2018). Implementing chemical, physics, biological, and physiochemical pretreatments has been used to increase the biodegradability of lignocellulosic waste, AD efficiency, and CH_4 yield (Cheng & Brewer, 2021).

Acidogenesis

Acidogenic microorganisms absorbed the soluble hydrolysis products through their cell membranes (Meegoda et al., 2018). Following different metabolic pathways small molecules are further degraded and fermented into volatile fatty acids (VFAs) (acetic acid, butyric acid, and propionic acid), organic acids (succinic acid and lactic acid), ammonia (NH_3), hydrogen gas (H_2), carbon dioxide (CO_2), hydrogen sulfide (H_2S), and low alcohols (Angelidaki et al., 2011).

In the acidogenic stage of AD, carbohydrates are converted into pyruvate (Sikora et al., 2018). Butyric acid fermentation (*Clostridium*-type fermentation) and mixed-acid fermentation (*Enterobacteriaceae*-type fermentation) are the main hydrogen-yielding fermentations in this process (Sikora et al., 2017). In *Clostridium*-type fermentation, Acetyl-CoA is converted to non-gaseous products including short-chain fatty acids (acetate, butyrate, lactate, and propionate), alcohols (ethanol but also butanol and propanol), and ketones (such as acetone) by a wide range of enzymes (Girbal et al., 1995). *Enterobacteriaceae*-type fermentation can include ethanol, short-chain fatty acids (formate, acetate, lactate, and succinate), as well as acetoin and 2.3-butanediol (Kraemer & Bagley, 2007).

The stoichiometric degradation pathways during fermentation of glucose can be described as the following equations (Weinrich & Nelles, 2021).

Table 1. Principal glucose degradation pathways.

Product	Reaction
Acetic acid	$C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ CH_3COOH + 2\ CO_2 + 4\ H_2$
Propionic acid	$C_6H_{12}O_6 + 2\ H_2 \rightarrow 2\ CH_3CH_2COOH + 2\ H_2O$
Butyric acid	$C_6H_{12}O_6 \rightarrow CH_3[CH_2]_2COOH + 2\ CO_2 + 2\ H_2$
Ethanol	$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2\ CO_2$
Lactic acid	$C_6H_{12}O_6 + H_2O \rightarrow 2\ CH_3CH\ OH\ COOH$

Source: (Weinrich & Nelles, 2021)

The acidogenesis of amino acids such as methionine, glycine, cysteine, leucine, isoleucine, threonine, valine, and serine are degraded to acetate, propionate, hydrogen, and carbon dioxide by Stickland reactions (Park et al., 2014), (Tang et al., 2015). In the Stickland reaction, different amino acids participate as electron donors or acceptors, in which the oxidation of one amino acid is bound to the reduction of another (Ramsay & Pullammanappallil, 2001). With single amino acids, degradation is also possible when hydrogenotrophic bacteria are present, although this process is known to be slower than the Stickland reaction (Sudalyandi & Jeyakumar, 2022).

Members of the *Clostridiales* (families *Clostridiaceae, Eubacteriaceae, Peptococcaceae, Peptostreptococcaceae*), *Fusobacterales, Synergistetes* (*Aminobacterium colombiense*) and *Cloacimonetes (Candidatus Cloacimonas acidaminovorans*) are capable of amino acid fermentation (Tang et al., 2015).

The main products of lipid hydrolysis are glycerol and long-chain fatty acids. In the acidogenic stage, glycerol can enter a reductive pathway and be converted to 1,3-propanediol or an oxidative pathway and be transformed to phosphoenolpyruvate (Sikora et al., 2017). On the other hand, long-chain fatty acids are broken down using the process of beta-oxidation, primarily producing acetic acid, whereas odd-chain fatty acids produce propionic acid (Weinrich & Nelles, 2021). Most microorganisms involved in hydrolysis are also involved in acidogenesis (Goswami et al., 2016). Other genera of anaerobic bacteria, such as *Propionibacterium* (propionic acid formation from carbohydrates), *Clostridium botulinum* (degradation of amino acids), and *Acetobacterium* (β - oxidation of fatty acids), have been identified (Deublein & Steinhauser, 2008).

One of the main characteristics of acidogenesis is its faster proceeding compared to other stages of anaerobic digestion (Deublein & Steinhauser, 2008). Also remarkable is the production of VFAs that create direct precursors for the final stage of methanogenesis (Akuzawa et al., 2011). However, high concentrations of hydrogen during this phase may cause inhibition of the production of acetate (Sayara & Sánchez, 2019). In addition, the acidification of VFAs causes methanogenesis stage inhibition due to the decrease of pH in the system (Li et al., 2010).

Acetogenesis

The resulting products from acidogenesis, hydrogen gas (H_2), carbon dioxide (CO_2), and acetate (CH_3COOH), can be directly metabolized by the methanogenic microorganisms responsible for the next stage (Fournier & Gogarten, 2008). However, other intermediates formed by acidogenesis (butyrate, propionate, valerate, caprionic acid, acid, and ethanol) must be additionally biodegraded into proper substrates (acetate, carbon dioxide, and hydrogen) to allow methanogenesis (Czatzkowska et al., 2020).

In the acetogenic stage, a syntrophic relationship between acetogenic and methanogenic bacteria takes place. Syntrophy is a special type of symbiotic cooperation between two metabolically diverse types of microorganisms that depend on one another for the degradation of a certain substrate (Sikora et al., 2017). As an example, hydrogenotrophic methanogens consume H_2 provided by homoacetogenic bacteria (Meegoda et al., 2018). The benefit to homoacetogenic bacteria is that their reactions require a low partial pressure of hydrogen to avoid metabolism inhibition (Tsapekos, 2017).

Under standard conditions, the oxidation of butyrate (Beta oxidation), propionate (Methylomalonyl-CoA), acetate (Wood-Ljungdahl), Lactato

(Lactate degradation), ethanol (Ethanol oxidation), and other non-gaseous products of acidogenesis occurs as the next stoichiometry of relevant degradation pathways (Sikora et al., 2017) (Weinrich & Nelles, 2021).

Table 2. Relevant degradation pathways during acetogenesis.

Substrates	Reactions	ΔG◦'(kJ/mol)
Propionate	$CH_3CH_2COOH + H_2O \rightarrow 2CH_3COOH + 3H_2 + CO_2$	+76.1
Butyrate	$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2$	+48.1
Lactate	$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2$	-4.2
Ethanol	$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2$	+9.6
Formate	$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$	-130
Acetate	$CH_3COOH \rightarrow CH_4 + CO_2$	-33
	Hydrogen utilizing reactions.	
Hydrogenotrophic methanogenesis	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-130.7
Homoacetogenesis	$4 H_2 + 2CO_2 \rightarrow CH_3COOH + H + 2H_2O$	-94.9
Sulphate reduction	$H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	-152.1

Source: (Weinrich & Nelles, 2021; Zhang et al., 2023)

The acetogenic bacteria are classified into homoacetogens and obligate hydrogen-producing acetogens. The first group produces acetate as its sole product (Drake et al., 1994). The most representative genres are *Clostridium, Acetoanaerobium, Acetobacterium, Acetogenium, Butyribacterium, Paleobacter, Treponema, and Halophaga* (Gerardi, 2003). Obligate hydrogen-producing *acetogens* primarily are *Syntrophomonas*, *Syntrophobacter*, *Syntrophospora,* and *Syntrophus (Czatzkowska et al., 2020)*.

Methanogenesis

Known as the final phase of the anaerobic digestion process. The biological formation of methane is performed by methanogens using three main substrates (Lackner et al., 2018). The first substrate is CO_2. Most methanogens can reduce CO_2 to methane using electrons from H_2. Other electron donors, such as formate and secondary alcohols such as 2-propanol, 2-butanol, and even ethanol, might also be used by methanogens. The second substrate is compounds that contain methyl groups, such as methanol and amines. The last group corresponds to acetate (Balagurusamy & Chandel, 2020).

The mentioned substrates are processed, respectively, through three recognized pathways of methanogenesis: hydrogenotrophic, methylotrophic (hydrogen-dependent and hydrogen-independent), and acetoclastic (Sikora et al., 2017).

1. Hydrogenophilic methanogenesis: Methane production by reduction of hydrogen and carbon dioxide.

$4H + CO_2 \rightarrow CH_4 + H_2O$ ($\Delta G^{0\prime} = -131$ kJ / mol)

$4HCOO^- + 4H^+ \rightarrow CH_4 + 3CO_2 + H_2O$ ($\Delta G_0{}^\prime = -145$ kJ / mol)

From this route, around 30% of methane may be produced (Lackner et al., 2018). The hydrogenotrophic pathway is much more energetically effective, and this may be one of the reasons for the dominance of the *Methanomicrobiales* (Sarker et al., 2019).

2. Methylotrophic methanogenesis: Methane production by decarboxylation of methyl alcohols, methylamines, and methyl sulfides (Lackner et al., 2018).

$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2\ H_2O$ ($\Delta G^{0\prime} = -105$ kJ / mol)

$CH_3OH + H_2 \rightarrow CH_4 + H_2O$ ($\Delta G^{0\prime} = -113$ kJ / mol)

Could be divided into species that use hydrogen (H_2) or work in their absence to reduce the methyl group to CH_4. As *Methanosarcinales*, *Methanomassiliicoccales*, and one species of *Methanobacteriales*. (Vanwonterghem et al., 2016).

3. Acetoclastic methanogenesis: Production of methane by acetate decarboxylation.

$CH_3COOH \rightarrow CH_4 + CO_2$

Most of the methane is produced by acetotrophic methanogens, which convert acetate into CH_4 and CO_2.

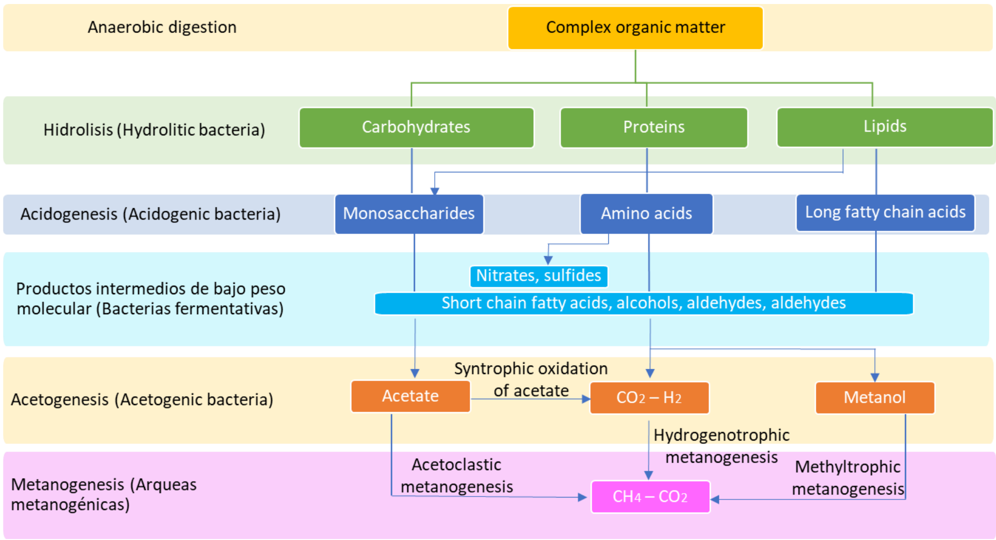

Figure 1. Characteristic phases during anaerobic digestion. (Arif et al., 2018; Li et al., 2021) with some modifications

2.1.3 Influence factors in anaerobic digestion

The anaerobic process is a complex process where any variation or fluctuation in the optimum operational conditions could affect or even inhibit methane production (Sayara & Sánchez, 2019). The relevant variables that influence the AD include feedstock type, reactor type, pH, temperature, retention time, organic loading, pressure, and inhibitory components (Sarker et al., 2019). The following factors must be periodically monitored and maintained within optimum ranges:

pH

The pH directly influences the growth and composition of the microbial community in anaerobic digestion. This parameter measures the acidity or alkalinity of a solution of a substrate mixture for the AD (Balagurusamy & Chandel, 2020). Depending on the concentration of individual acids and bases, as well as the existing buffer system, the concentration and activity of free hydrogen ions and pH value change (Elefsiniotis & Oldham, 1994).

Fluctuations in pH during the various stages of AD are common due to the diverse metabolic pathways of the microbial community. While the acid-forming bacteria prefer slightly acidic conditions (pH 5.9-6.6), the optimal pH for methanogens is within the neutral range (pH 7.2-8.0) (Sudalyandi & Jeyakumar, 2022).

Alkalinity

Alkalinity reflects the buffering capacity and maintains the proper pH for the digester content. Alkalinity is presented in the form of bicarbonates that exist in equilibrium with released carbon dioxide at a given pH (Meegoda et al., 2018).

In an anaerobic digestion process, alkalinity is influenced by carbon dioxide and carbonate ions since ammonia released by the decomposition of nitrogen-rich feedstocks with high proportions of proteins and amino acids can react with dissolved carbon dioxide (Schnurer & Jarvis, 2010). When the pH is around 7.0, as is often the case in anaerobic reactors, the alkalinity is equivalent to the concentration of bicarbonate, hydrogen sulfide, dihydrogen phosphate, or ammonia, which buffers the system in the optimum pH range for the process to run efficiently (Spanjers, 2006).

Temperature

The temperature has a three-dimensional impact on the AD system since it influences microbial growth rate, complicated substrate hydrolysis, and enzymatic action (Sudalyandi & Jeyakumar, 2022). The temperature tolerance of microorganisms has been categorized as psychrophilic (4-25 °C), mesophilic (30-40 °C), thermophilic (50-60 °C), or extremophilic (65 °C) (Bramstedt, 2016).

As an essential parameter that can affect microbial growth, different efforts have been made to study the influence of temperature on biogas production. Kougias et al. (2017) demonstrated that thermophilic conditions produce more methane and CO_2 than mesophilic conditions do. However, Mesophilic bacteria are more robust and can tolerate higher changes in environmental parameters, including temperature. Hence, the biodegradability process requires a longer retention time and yields less biogas. On the other hand, mesophilic digesters remain attractive because of their stability and lower heating energy costs compared to thermophilic digesters (Alrowais et al., 2023).

C: N *ratio*

The carbon-nitrogen ratio of organic matter shows the relationship between carbon and nitrogen, and it is an indicative measure of the nutrient balance that anaerobic organisms require for growth (Verma, 2002).

An optimum C:N ratio in AD ensures a sufficient nitrogen supply for cell production and the degradation of the carbon present in the feedstock. In most cases, the C:N ratio varies between 10:1 and 30:1 (Khanal et al., 2019). The optimum combination of sargassum and pig manure was described by Rivera-Hernández et al. (2022). On the other hand, an excess amount of some nutrients may become inhibitory to the AD process (Chen et al., 2008).

Volatile fatty acids (VFAs)

The performance of the AD process is affected by the concentration of intermediate products such as VFA (e.g., acetate, propionate, and butyrate), produced during acidogenesis (Ferranti et al., 2018). An increase in their concentration over the acceptable range can lead to pH value reductions and an unbalanced digestion process. If the digester has sufficient buffer capacity, the accumulation of VFAs will not necessarily result in pH value reductions. Due to this, there could be a concentration of VFA optimal for one type of digester but inhibitory for another type. Finally, the VFA concentration cannot be recommended as a stand-alone process monitoring parameter (Al Seadi et al., 2013).

2.1.4 Batch and continuous anaerobic digestion processes.

Based on the feeding method, anaerobic digestion can be classified as batch, semi-continuous, or continuous (Forster-Carneiro et al., 2008).The single-stage batch reactors are comparatively simple to operate and robust in nature. A major advantage of a single-stage digester is its elevated reaction rate. Since the reaction rate of single-stage batch reactors is high, they yield remarkably considerable amounts of methane (Gonzalez-Martinez et al., 2016). Considering that continuous setup is more laborious and time-consuming than batch tests, the latter have been more widely used. It is important to note that the batch approach can be used for three purposes: anaerobic biodegradability, inoculum activity, and inhibition (Rozzi & Remigi, 2004).

High gas production rates in the first hours and days of an experiment are used to identify rapidly degradable substrate components (such as dissolved sugars and amino acids), whereas a low gas production rate at the end of the experiment provides information on slowly degradable constituents (Girault et al., 2012). A reasonable application of this method strongly depends on the characteristic gas production curves of individual substrates and the accuracy (and transferability) of the applied batch tests (Weinrich & Nelles, 2021). On the other hand, semi-continuous operation refers to feeding and discharging materials within the specified residence time, so that the matrix concentration of the reactor is always maintained at the same level (He et al., 2022).

2.1.5 Biochemical methane potential

The biochemical methane potential (BMP) test is an anaerobic digestion carried out normally in batch reactors for a prolonged time. The main objective of this test is to estimate the ultimate biomethane or biogas potential of a specific substrate (Tassew et al., 2019).

In historical terms, the current BMP method of incubating samples with defined media and inoculum under controlled conditions arose from the challenges faced with evaluating anaerobic biodegradability using respirometer batch procedures (Owen et al., 1979).

In BMP assays, there is no defined volume for batch reactors, but volumes of 0.5–1 L are often used. To evaluate the complete process in this test, substrate and inoculum used during anaerobic digestion are characterized by total and soluble chemical oxygen demand (COD), total solids (TS) volatile solids (VS), and various other parameters. Biochemical methane potential tests are widely used due to their low cost, simplicity, and repeatability. Even though BMP tests take a long time, usually longer than 30 days (Sawatdeenarunat et al., 2015).

Results of BMP assays are dependent on conditions such as temperature and pressure, inoculum, the concentration of substrate, inoculum to substrate ratio (ISR), stirring, and composition of flush gas (Wang, 2016).

2.2 Anaerobic co-digestion

Co-digestion refers to the addition of a second type of waste to improve the nutrient balance and increase methane production (Balagurusamy & Chandel, 2020). The co-digestion of substrates can improve biogas yield during AD in various combinations and fractions. According to Lehtomäki et al. (2007) methane production increased by up to 65% by using co-digestion of energy crops and crop residues with cow manure.

Major benefits of co-digestion can be improvements in the C:N ratio of the feedstock, thereby improving overall biodegradation and methane production and improving economics through waste diversification (Ashekuzzaman & Poulsen, 2011).

2.2.1 Anaerobic digestion of sargassum

Sargassum spp. is a macroalgae belonging to the group of brown algae (*Phaeophyta*) that inhabit the seas around the world. Its genus includes more than 300 species both benthic and holopelagic, the latter can aggregate to form extensive floating masses on the sea surface, within which it is possible to find dozens of fish species in stages from larval to adult (Farrell et al., 2014).

The physicochemical components of sargassum; were studied by (Vázquez, 2021) because of analyzing the sargassum species that arrived off the Mexican Caribbean coast between September 22nd and 23rd, 2018. Their characterization found high moisture content, carbohydrate-rich, negligible lignin and low cellulose content. This unique composition supports the microbial conversion of organic matter to biogas. (Amador-Castro et al., 2021; Thompson et al., 2021)

Nonetheless, methane varies widely due to variation between sargassum species, location and seasonal chemical composition of the biomass (Milledge et al., 2020). A cost-effective alternative is the co-digestion of sargassum with various kinds of carbon-rich waste such as pig manure (Rivera-Hernández et al., 2022), glycerol and frying oil (Oliveira et al., 2015), and food waste (Thompson et al., 2021), as co-substrates.

Table 3. Biochemical composition of Sargassum biomass.

Parameter	Sargassum collected on Playa delfines Cancún.
Proximate composition (%)	
Dry weight	17 ± 1.2
Ash	20.7 ± 0.6
Carbohydrate	14.1 ± 1.8
Protein	8.1 ± 0.7
Lipids	3.3 ± 0.6
Elemental analyses (%)	
Carbon	30.1 ± 1.2
Nitrogen	1.1 ± 0.1
C/N ratio	27.3 ± 1.2
Pigments and phenols	
Chlorophyll a (mg·g^{-1} dw)	0.13 ± 0.03
Chlorophyll c (mg·g^{-1} dw)	0.04 ± 0.01
Carotenoids (mg·g^{-1} dw)	0.08 ± 0.02
Phenols (µg·mL^{-1})	7.39 ± 1.06
Cell wall polysaccharides (%)	
Fucoidan	8.5 ± 1.4
Alginate	30.5 ± 1.7
Uronic acids from alginate	24.4 ± 1.1
Uronic acids from fucoidan	11.9 ± 0.6
Sulfates from fucoidan	14.3 ± 0.7
Metals (mg·kg^{-1})	
Cd	0.32 ± 0.32
Cu	1.09 ± 1.61
Fe	25.9 ± 6.88
Pb	0.29 ± 0.33
Zn	5.03 ± 1.53
As	64.90 ± 5.29

For quantitative data ± SD is shown. DO: Dissolved oxygen, POM (Particulate organic matter) TDS: total dissolved solid.
Source: (Vázquez, 2021)

2.2.2 Anaerobic digestion of pig manure

The physicochemical components of pig manure are fully described in the study conducted by Antezana et al. (2016), because of the analysis of a total of 77 samples of pig manure from farms located in Central and Eastern Spain. One of the most relevant results for the focus of this research was the C: N ratio, whose mean in manure from fattening pigs was 4.18 value that contrasts with the result of 10.4 found by (Aust et al., 2009).

The difference in both results is due to the variability in terms of housing systems, feed composition, climate or type of farm (Beccaccia et al., 2015).

Table 4. Physicochemical characterization of pig slurry.

Physicochemical characteristics	All samples n=77	
	Mean	CV
pH	7.52	5.5
Dry matter ($kg{\cdot}m^{-3}$)	49.3	78.9
Organic matter ($kg{\cdot}m^{-3}$)	36.7	82.8
Ash (kg·m-3)	12.5	70.7
Macronutrients		
N ($kg{\cdot}m^{-3}$)	4.79	64.5
P ($kg{\cdot}m^{-3}$)	0.86	90.9
K ($kg{\cdot}m^{-3}$)	1.66	64.3
Ca ($kg{\cdot}m^{-3}$)	1.23	95.9
Mg ($kg{\cdot}m^{-3}$)	0.56	84.1
S ($kg{\cdot}m^{-3}$)	0.30	74.7
Other characteristics		
C ($kg{\cdot}m^{-3}$)	20.20	81.3
TAN[1] ($kg{\cdot}m^{-3}$)	3.07	64.6
Organic N ($kg{\cdot}m^{-3}$)	1.67	87.9
VFA[2] ($mol{\cdot}m^{-3}$)	75.4	106.1
Acetic ($mol{\cdot}m^{-3}$)	47.2	112.3
Propionic ($mol{\cdot}m^{-3}$)	14.9	100.8
Butyric ($mol{\cdot}m^{-3}$)	6.49	164.9
Ratio acetic/propionic	6.42	117.6
Ratio acetic/butyric	53.7	134.8
Ratio butyric/propionic	0.35	96.2
Ratio C: N	4.05	53.9
Ratio N:P	7.82	61.8
Ratio N: K	3.15	36.8

[1]TAN: total ammonia nitrogen, [2]VFA: volatile fatty acids

Source: (Antezana et al., 2016)

The physicochemical characteristics of pig manure show high micronutrient levels, buffering capacity, and biodegradability, which allow the growth of anaerobic microbes. However, high concentrations of volatile solids (VS) and total solids (TS) (greater than 20%) also lead to excessive organic acids and ammonia, which seriously inhibit methanogenesis (Duan et al., 2019).

2.3 Magnetite nanoparticles in anaerobic digestion

Anaerobic digestion performance can be improved using Nanotechnology. Nanoparticles (1–100 nm) have excellent physicochemical properties, such as high activity, high reactive surface area, chemical stability, high specificity for improving performance, and the ability to stimulate microbial growth in the AD (Cao & Wang, 2004; Zhang et al., 2021).

Magnetite (Fe_3O_4) is a common, low toxicity, ferromagnetic, black-colored iron oxide that is widely distributed on earth (Fu et al., 2019). Fe_3O_4 supplementation in anaerobic digestion processes is an inexpensive, economic, and simple method to synthesize the nanoparticles, allowing large-scale utilization (Boscaro et al., 2022).

Many authors have reported an increase in methane production using Fe_3O_4 as an additive in anaerobic digestion (Table 5). finding that magnetite could mediate direct interspecies electron transport between the *Geobacter* species and the *Methanosarcina* species to promote methane production (Kato et al., 2012). Besides, Yin et al. (2018) found that the continuous addition of Fe_3O_4 in syntrophic methanogenic communities could induce acetoclastic methanogens to become hydrogenotrophic methanogens and promote the syntrophic interaction between bacteria and methanogens.

Table 5. Magnetite nanoparticles in anaerobic digestion.

Substrate	Inoculum	Experimental conditions	CH_4 yield (mL $CH_4 \cdot g_{VS}^{-1}$)	Nanoparticles Characteristics	Reference
Waste activated sludge.	Wastewater	Waste activated sludge (WAS) Primary sludge (PS)	161 177 203 273 205 157 180 194 231	Control 40 mg·L^{-1} 12-18 nm 80 mg·L^{-1} 12-18 nm 120 mg·L^{-1} 12-18nm 250 mg·L^{-1} 12-18 nm 40 mg·L^{-1} 50-100 nm 80 mg·L^{-1} 50-100 nm 120 mg·L^{-1} 50-100 nm 250 mg·L^{-1} 50-100 nm	Hassanpourmoghadam et al. (2023)
Cattle manure	Cattle manure	Concentrations of Fe NPs: 15, 30, and 60 mg/L Diameter: 435.1 nm.	18.59 31.81 40.68 37.81	Control 15 mg·L^{-1} 435.1 nm 30 mg·L^{-1} 435.1 nm 60 mg·L^{-1} 435.1 nm	Abdelwahab et al. (2022)
Crude glycerol	Poultry wastes	Concentration of Magnetite (Fe_3O_4) nanoparticle: (200 mg/L, 400 mg/L, 800 mg/L) Size: 20 nm approx.	579.6 868.8 744.8 731.2	Control 200 mg·L^{-1} 20 nm 400 mg·L^{-1} 20 nm 800 mg·L^{-1} 20 nm	Boscaro et al. (2022)

Dairy manure Corn Stover	Wastewater	Pretreatment: 509 mL of 8% solution of calcium hydroxide (Ca $(OH)_2$) Concentrations of Magnetite (Fe_3O_4) nanoparticle: 20, 50, and 75 mg·L^{-1} Size: 15–20 nm	178 191 178 174	Control 20 mg·L^{-1} 20 nm 50 mg·L^{-1} 20 nm 75 mg·L^{-1} 20 nm	Ajayi-Banji and Rahman (2021)
Chicken litter	Chicken litter	Concentration of Magnetite (Fe_3O_4) nanoparticle:(20 mg·L^{-1}, 40 mg·L^{-1}, 60 mg·L^{-1}) Size: 4.2 nm.	73.29 126.42 108.33 78.85	Control 20 mg·L^{-1} 4.2 nm 40 mg·L^{-1} 4.2 nm 60 mg·L^{-1} 4.2 nm	Aguilar-Moreno et al. (2020)
Waste sludge	Wastewater	Concentrations of Fe_3O_4 NPs: 0, 20, 50, 100, 200 mg·L^{-1} Diameter: 20-30 nm	68.1 75 94.9 109.8 91.4	Control 20 mg·L^{-1} 30 nm 50 mg·L^{-1} 30 nm 100 mg·L^{-1} 30 nm 200 mg·L^{-1} 30 nm	Zhang et al. (2020)

2.3.2 Syntrophic relationships and direct transmission of electrons between microorganisms

Tian et al. (2014) define syntrophy as a form of symbiosis between two metabolically diverse groups of bacteria that allows the degradation of various substrates. The process of anaerobic digestion is the result of three groups of microorganisms working together, grouped according to the process they perform. Primary bacterial fermentation, in which hydrolytic and acidogenic microorganisms the acetogenesis developed by acetogenic syntrophic bacteria, and finally the methanogenesis carried out by methanogenic archaea (Gerardi, 2003).

Direct interspecies electron transfer (DIET) is a relevant mechanism by which syntrophic microorganisms join forces to oxidize organic matter and reduce CO_2 to CH_4 (Batstone et al., 2006). An inefficient interspecies electron transfer between syntrophic bacteria and methanogens could restrict the reaction rates and efficiency of anaerobic digestion. Direct interspecies electron transfer (DIET) has been proposed as a pathway that can improve AD performance by increasing electron transfer efficiency (Madigan et al., 2012).

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3. METHANE PRODUCTION FROM PIG MANURE IN CO-DIGESTION WITH SARGASSUM AND USE OF NANOPARTICLES

3.1 Introduction

Annually, inundations of *Sargassum* spp. arrive in widespread coastal areas of the Atlantic Ocean (Salter et al., 2020). There are several factors contributing to this phenomenon. The increment of ammonium NH_4^+ concentrations on the Amazon continental shelf are due to anthropic activities such as excessive use of agrochemicals, deforestation, livestock, and urban sewage (Aquino et al., 2022). Global climate change and rising sea surface temperatures are among the factors that increase sargassum blooms. Resulting in expanding ecological crises (Liu et al., 2021).

The massive arrival of *Sargassum* spp. has occurred on the coasts of the Mexican Caribbean since the summer of 2014, covering 2,360 m^3 of sargassum per kilometer of beach (Rodríguez-Martinez et al., 2016). Additionally, Chávez et al. (2020) reported the movement of sargassum in the open sea off the Mexican coast from Cancun, in the north, to Xcalac, in the south. From January 2016 through August 2020, this pelagic macroalga covered about 22,900 ha of ocean surface in September 2018 and about 19,000 ha in April 2019. Furthermore, the total amount removed from seven Mexican Caribbean beaches during 2018 and 2019 was 181,081 m^3 and 203,269 m^3 respectively (Rodríguez-Martinez et al., 2022). This situation has affected the region, generating social, economic, and environmental issues. Such as negative effects on the composition of coastal water (Camacho-Cruz et al., 2022), the formation of brown tides near the coast, resulting from the decomposition of organic matter (Chávez et al., 2020), and excessive costs for sargassum removal, estimated at USD 210 million (Davis et al., 2021).

Several other coasts all over the world have experienced similar or worse impacts than the ones mentioned before. For this reason, researchers in the affected areas have proposed strategies to integrate these coastal wastes into a circular economy. *Sargassum* spp., from Guadeloupe (French West Indies), was useful in synthesizing activated carbon for the removal of organic pollutants from contaminated water (Francoeur et al., 2022). In Côte d'Ivoire, Ouattara et al. (2022) characterized and cataloged the phenolic compounds present in *Sargassum natans* and *fluitans* as food additives with antioxidant properties. In Spain, *Sargassum muticum* was evaluated as part of a novel bio-refinery scheme to produce plant bio-stimulant, biogas, and adsorbents for pollutant removal and mineral amendments (Flórez-Fernández et al., 2021).
Finally, Mexico has made a multisectoral effort to change the perspective of sargassum from "national problem" to "national resource." Considering and fully understanding the ecological importance of this macroalgae as a floating ecosystem and its potential as an economic resource once it arrives massively to Mexican coastal areas (Rosellón-Druker et al., 2022). In fact, Rivera-Hernández et al. (2022) proposed the co-digestion of sargassum-pig manure for methane production.

Anaerobic digestion (AD) is a biotechnological process for waste biomass utilization and energy conservation (Xu et al., 2022). The use of seaweed has been successfully investigated as a substrate for this degradation process (Farghali et al., 2021; Thakur et al., 2022; Yuhendra et al., 2021). However, the biochemical composition of algal biomass causes low methane yields in biogas production. Thompson et al. (2019) described the low bioconversion of brown seaweeds due to the inaccessibility of complex sugars for microbial degradation. Other inhibitory factors are the metalloids and metals bioaccumulated by sargassum and other seaweeds (Robledo et al., 2021). According to Nielsen et al. (2021), *Sargassum* spp. may need to be pre-treated prior to AD or co-digested with other waste biomass to increase biochemical methane potential.

The biogas composition and quantity can be enhanced not only through operating conditions and substrate type (Bharati & Suresh, 2017), but also by adding nanomaterials to AD systems, which can improve their performance and stability (Junjie Li et al., 2021). As electron donors or acceptors, nanoparticles (NPs) could promote biogas production by strongly degrading biomass by direct interspecies electron transfer (Cerrillo et al., 2021; Zhang et al., 2023).

By including nanotechnology in the anaerobic digestion process. The nano-sized structures with specific physicochemical properties interact with the substrate and microorganisms (Jadhav et al., 2021). Some applications of this nanotechnology have made it possible to maximize methane yield from the anaerobic digestion of seaweed, including nanoparticles of magnetite Fe_3O_4 (El Nemr et al., 2021), cobalt (Zaidi et al., 2023), and modified biochar in combination with iron oxide Fe_2O_3 (Zaidi et al., 2019). The material they are made of, as well as their size and concentration, determine their performance (Hassanpourmoghadam et al., 2023).

Previous studies have reported the highest methane production due to the addition of 20 $mg{\cdot}L^{-1}$ magnetite nanoparticles (MNPs) to anaerobic digestion. Abdelsalam et al. (2017) achieved 351.8 mL $CH_4{\cdot}g_{VS}^{-1}$. Aguilar-Moreno et al. (2020) achieved 126.42 mL $CH_4{\cdot}g_{VS}^{-1}$. On the other hand, Zhang et al. (2020) reported 75 mL $CH_4{\cdot}g_{VS}^{-1}$. Considering that, the co-digestion process of pig manure and *Sargassum* spp. reported an improvement in BMP from 79.5 to 160.4% with respect to mono-digestion treatments (Rivera-Hernández et al., 2022). The purpose of this study was to investigate the influence of magnetite nanoparticles at 20 $mg{\cdot}L^{-1}$ on methane production during the co-digestion of pig manure and sargassum. A modified Gompertz model (MGM) was used to estimate biochemical methane potential from experimental data. To understand the anaerobic digestion stages, volatile fatty acid (VFA) profiles were analyzed.

3.2 Material and methods

3.2.1 Nanoparticles synthesis

Magnetite Fe_3O_4 nanoparticles were synthesized according to the chemical co-precipitation method used by Aguilar-Moreno et al. (2020), based on the modified methodology proposed by (Abdullah et al., 2017). The reagents used for the synthesis of the Fe_3O_4 were ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) (Meyer®) as precursors, gelatin (Sigma Aldrich®) as passivating agent, NaOH (JTBaker®) for pH adjustment, and deionized water (18 $M\Omega \cdot cm^{-1}$, Easypure) as solvent. The major net chemical reaction can be written as follows (Szalai et al., 2019):

$$\mathrm{FeCl_2 + 2FeCl_3 + \ NaOH \ \rightarrow Fe_3O_4 + 8Na^+ + 8Cl^- + 4H_2O}$$

Gelatin (1.5 g) was dissolved in deionized water, heated for 30 min, and sonicated (EW-08895-91, Cole-Parmer®, USA). Subsequently, $FeCl_3$ and $FeCl_2$ salts were added (molar ratio), and the solution was subjected to a heat treatment (60 °C) for 30 min; after this time, NaOH was added until the pH of the solution was adjusted to 11, and after 30 min, the mixture was sonicated for 45 min. The obtained solution was centrifuged at 6,700 rpm (SL 16R, Thermo Fisher Scientific™, Germany) for 10 minutes and washed three times with deionized water.

3.2.2 Characterization of nanoparticles

Hydrodynamic diameter (HD) and zeta potential characterization were determined with a nanozetasizer (ZEN 3600, Malvern Instruments, UK). To perform the analyses, the NP solution was diluted 1:40 with deionized water and sonicated for 20 min.

Size morphology and electron diffraction of the NPs were determined by transmission electron microscopy (TEM; Tecnai F30, FEI®, USA) operated at an accelerating voltage of 300 kV. The preparation of the sample consisted of diluting 1:20 the NP solution with deionized water and sonicating it for 10 min; subsequently, one drop of the dilution was placed on a carbon-coated copper grid and left to dry at room temperature for 24 h.

The X-ray powder diffraction (XRD) technique was used to identify the crystalline phases of the specimen and measure its structural properties. Prior to the analysis, the NP solution was dried at 60 °C for 24 h. The analysis of the synthetized MNPs (magnetite nanoparticles) was obtained with a diffractometer (D8 Advance, Bruker®, Germany), with Co Ka (k¼ 1.5405 Å, 40 kV and 40 mA) radiation source, in the 2θ range of 10°- 80°, with step size and scan velocity of 0.05° and 3 s, respectively.

3.2.3 Raw material and collection sites

A mixed sample of brown macroalgae, *Sargassum* spp., was collected from the Cancun, Quintana Roo coast (21°03′ 17.0 " N 86°46′ 49.6 "W) at the seashore of the coastal area. Sample preservation was carried out in a cold store at -20 °C, dried at 37 °C for 48 hours, and finally milled.

The pig manure was collected from an experimental farm located in Chapingo, Estado de Mexico, Mexico. The sample preservation was carried out in a cold store at 4 °C. The slurry preparation was obtained by adding water to pig manure and filtering the resulting solution to remove large particles.

The elemental analysis of sargassum was 89.80% TS, 50.70% VS DB (Dry Basis) carbon 27.10%, hydrogen 3.8%, nitrogen 1.20%, and sulfur 0.90%. In addition, pig manure analysis was 27.20% TS (DB), 80.80% VS (DB), carbon 37.50%, hydrogen 5.7%, nitrogen 3.40%, and sulfur 0.70%, which were reported by our research group (Rivera-Hernández et al., 2022).

3.2.4 Experimental Procedure

In serological bottles (305 mL total volume) used as microcosms, a batch experiment was conducted. Slurry was prepared using *Sargassum* spp. (S) and pig manure (PM), having a target concentration of 3.0% of total solids (TS). Accordingly, S: PM ratios were determined for each substrate. 0:100 (0S-100PM), 15:85 (15S-85PM), 30:70 (30S-70PM), 50:50 (50S-50PM), 65:35 (65S-35PM), and 100:0 (100S-0PM). All treatments were prepared in triplicate and tested in the presence of 20 $mg \cdot L^{-1}$ of magnetite nanoparticles in accordance with Aguilar-Moreno et al. (2020). Inoculum was obtained from an active bio-digester that was fed chicken litter at 3% TS for five years and enriched with sodium propionate at 30 $g \cdot L^{-1}$. The microcosms were created with an initial working volume of 230 mL, and anoxic water was used to achieve a 3.0% TS for each treatment. Then hermetically sealed with a rubber septum and metal cap. Oxygen was displaced from the microcosms using a nitrogen gas purge. The BMP test was conducted for 100 days at 37 °C ± 1. Initial conditions are reported in Table 6.

Table 6. Initial Characterization of treatments.

Parameters		C: N	pH	TS (%)	VS/TS (%)	COD ($mg \cdot L^{-1}$)	Alkalinity ($mg \cdot L^{-1}$)	Sulfide ion ($mg \cdot L^{-1}$)	Sulfate ion ($mg \cdot L^{-1}$)
Treatments	**100 PM**	11	8.18	3.02 ± 0.1^{a}	71.83 ± 0.7^{a}	$40{,}461 \pm 1292^{a}$	$7{,}400 \pm 145^{a}$	$2{,}883 \pm 38.6^{a}$	$5{,}971 \pm 11.3^{a}$
	15S-85PM	12.8	8.02	3.20 ± 0.2^{a}	67.49 ± 1.9^{a}	$37{,}461 \pm 1575^{a}$	$7{,}011 \pm 126^{a}$	$2{,}811 \pm 13.6^{a}$	$5{,}851 \pm 22.2^{a}$
	30S-70PM	14.5	7.88	2.97 ± 0.1^{a}	60.99 ± 1.6^{b}	$33{,}211 \pm 1410^{b}$	$5{,}289 \pm 168^{b}$	$2{,}290 \pm 20.5^{b}$	$4{,}465 \pm 83.4^{b}$
	50S-50PM	16.8	8.09	3.28 ± 0.2^{a}	53.34 ± 2.5^{b}	$30{,}156 \pm 647^{bc}$	$4{,}822 \pm 150^{c}$	$2{,}021 \pm 54.9^{c}$	$3{,}908 \pm 63.8^{c}$
	65S-35PM	18.5	8.09	3.07 ± 0.2^{a}	49.21 ± 3.7^{cd}	$27{,}100 \pm 1167^{cd}$	$4{,}344 \pm 126^{d}$	$1{,}601 \pm 19.8^{d}$	$2{,}947 \pm 43.0^{d}$
	100 S	22.6	8.07	3.13 ± 0.2^{a}	46.77 ± 1.1^{d}	$24{,}239 \pm 1254^{d}$	$2{,}044 \pm 150^{e}$	693 ± 65.7^{e}	$2{,}241 \pm 83.4^{e}$

TS: total solids; VS: volatile solids; COD: chemical oxygen demand. The data are the average of three replicates with standard errors.
Means with the same letter in the same row do not present significant statistical difference, Tukey ($p<0.05$)

3.2.5 Analytical Methods

Physicochemical parameters such as total solids (TS), volatile solids (VS), alkalinity, chemical oxygen demand (COD), sulfide, and sulfate ions were determined at the beginning and end of the experiment by following the standard APHA methods (APHA AWWA, 1998). The pH values were determined using a potentiometer (Orion 5 Star, Thermo Fisher Scientific™, Singapore).

The volatile fatty acid (VFA) profile of each treatment was determined for acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate, iso-caproate, caproate, and heptanoate. Using a gas chromatograph (Claurus 500, PerkinElmer®, USA) equipped with a flame ionization detector (FID) and an Elite-FFAP capillary column (PerkinElmer®, USA, 30 m × 0.32 mm × 0.25 µm). The following chromatographic conditions were used: gas flow of 3.2 mL min^{-1} at 10.6 psi, 160 °C injection port, 80 °C oven for 1 min with a ramp of 160 °C for 8 min, 15°C·min^{-1} increase up to 250 °C detector, and retention time of 14 min. Nitrogen gas was used as the carrier gas. Free Volatile Acid Mix, analytical grade (CRM-46975, Brand Sigma-Aldrich, USA) was used as a standard. The change of VFA concentration with respect to time was calculated using declivity formula (annex).

Biogas volume production and methane concentration were monitored daily by the displacement method in 10% (v/v) saline water, whereas the methane concentration was measured from 10 µL of biogas obtained from each microcosm injected into a gas chromatograph (Claurus 500, PerkinElmer®, USA). The operating temperature conditions of the injection port, detector and oven were 100, 150 and 70 ◦C, respectively. Methane percentage was obtained by a calibration curve with pure methane (HDSP No. P-4618-F, Praxair®, Mexico), and it is reported under standard pressure and temperature conditions. Both parameters were evaluated daily.

3.2.6 Modeling and statistical analysis

The process dynamics of AD were investigated to estimate biodegradability differences between control and co-digestion substrates. SigmaPlot version 13 was used to model the modified Gompertz (Sweltering et al., 1990).

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

Where AMY is the accumulated methane yield (mL $CH_4 \cdot g_{vsf}^{-1}$) over time (t)

BMP – Biochemical methane potential (mL $CH_4 \cdot g_{vsf}^{-1}$)

e – Mathematical constant (2.718282)

μ_m – Methane production rate (mL $CH_4 \cdot g_{vsf}^{-1} \cdot d^{-1}$)

λ – Lag phase (d)

The mentioned parameters obtained with the modified Gompertz model (BMP, μ_m, and λ) were evaluated by ANOVA and a further comparison of means was performed by the Tukey test (SAS System 9.0) to determine significant statistical differences among treatments.

3.3 Results and discussion

3.3.1. Characterization of nanoparticles

Dynamic Light Scattering (DLS) combined with Zeta Potential (ZP) are specific techniques to study nanoparticle behavior in suspensions and provide information about the hydrodynamic size and surface charge of NPs, respectively (Bhattacharjee, 2016). Particularly, DLS is a non-destructive technique in which the scattered light is used to measure the diffusion speed of the particles, and the data of the movement of the particle in the liquid are processed to determine the hydrodynamic diameter (HD) (Hulst & van de Hulst, 1981) (El-Saadony et al., 2021). Movements will be faster for smaller particles and slower for larger particles (Stetefeld et al., 2016). The hydrodynamic diameter of synthesized magnetite nanoparticles (Fe_3O_4) in this study was 75.21 nm (annex).

The ZP, also termed electrokinetic potential, is the measurement of stability, charge, and the ability of nanoparticles to aggregate (Chellapandian et al., 2019). Its value reflects the potential difference between the EDL (electric double layer) of electrophoretically mobile particles and the layer of dispersant around them at the slipping plane (Bhattacharjee, 2016). In this case, the zeta potential value obtained was -11.8 (mV) (annex).

Transmission electron microscopy (TEM) was used to characterize magnetite nanoparticles, Fe_3O_4. The micromorphologies showed spherical clusters of nanoparticles (detail in Fig. 2A). Fig. 2B shows that the average size of 100 NPs was 6.10 ± 1.14 nm. These results are comparable to those obtained by Aguilar-Moreno et al. (2020) reported a size of 4.20 ± 0.73 nm, using the co-precipitated method and gelatin as a passivant agent. X-ray diffraction (XRD) was used to complement the nanoparticles characterization. It can be seen in Fig. 1C the crystalline structure of the magnetite nanoparticles by well-defined peaks with Bragg reflections at 2θ = 18.17° (111), 30.28° (220), 35.54° (311), 43.00° (400), 53.86° (422), 57.27° (511), 62.93° (440), 70.74° (620), and 74.59° (533), which correlate with the cubic phase (CP) of magnetite NPs (Fe_3O_4). The most intense peak corresponds to the plane (311), which agrees with the crystallographic pattern of the structures (letter number PDF 00-089-0691, ICDD). Therefore, it can be inferred that the MNPs synthesis process has good crystallinity.

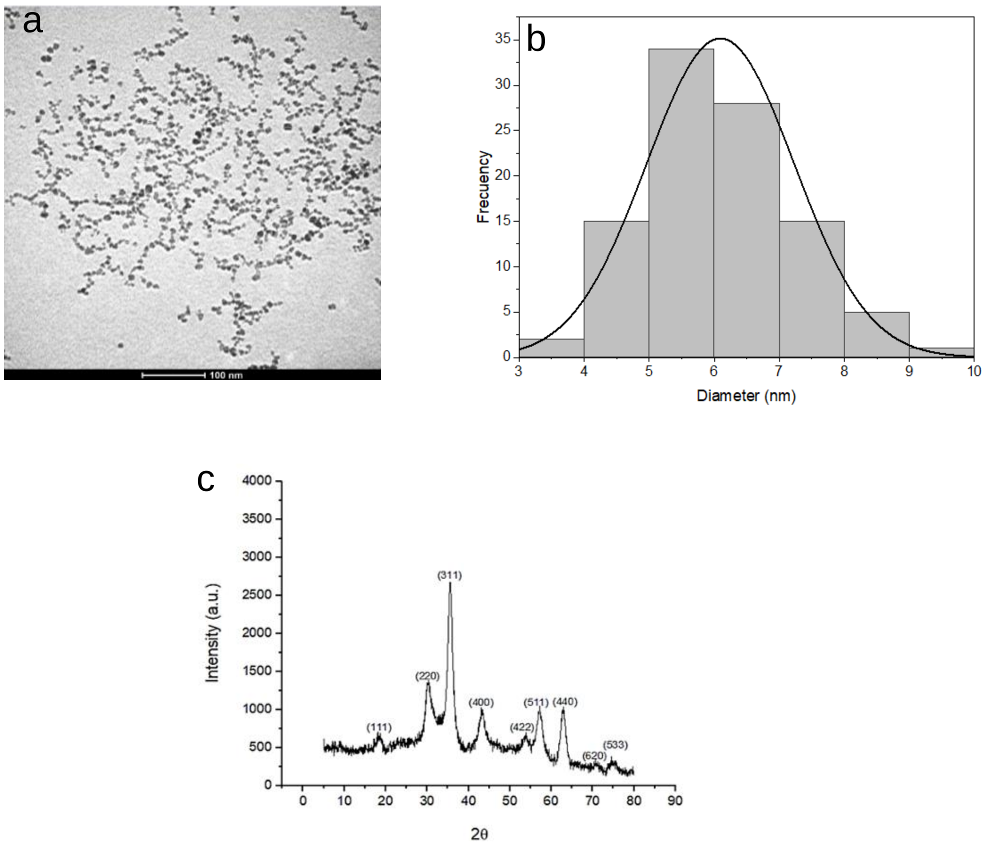

Figure 2. Synthesized magnetite nanoparticles.
a) Image in transmission electron microscopy. b) Histogram of size distribution of 100 nanoparticles. c) X-ray diffraction pattern of magnetite nanoparticles.

3.3.2 Influence of MNPs on biogas yield

Magnetite nanoparticles have been successfully used to enhance biogas production and methane content in anaerobic digestion (Kassab et al., 2020) (Boscaro et al., 2022). The yield of methane and biogas varies depending on the NPs used as well as the size and concentration added to the anaerobic digestion process (François et al., 2023). The daily biogas production of the experiment is shown in Figure 3b. The co-digestion treatments and the control of pig manure were higher in comparison with the control of sargassum. Rapid biogas production started after 10 days in 0S-100PM, 15S-85PM, 30S-70PM, 50S-50PM, 65S-35PM treatments. The maximum daily peak production for the co-digestion treatments was found in the 65S-35PM treatment generating 238 mL on 23 days. In the other treatments, the maximum daily biogas production was as follows, 195 mL on 23 days (30S-70PM), 193 mL on 16 days (50S-50PM), 156 mL on 14 days (65S-35PM) and finally 45 mL on 33 days (100S-0PM).

The cumulative biogas yield obtained for different C: N concentrations of sargassum and pig manure dosed with 20 $mg \cdot L^{-1}$ of magnetite nanoparticles is shown (Figure 3b). It can be observed that the highest biogas production was achieved with co-digestion treatments 30S-70PM (907.8 ± 14.5 $mL \cdot g_{VS}^{-1}$) and 50S-50PM (899.5 ± 25.7 $mL \cdot g_{VS}^{-1}$). Followed by 65S-35PM (859.5 ± 54.2 $mL \cdot g_{VS}^{-1}$), and 15S-85 PM (802.4 ± 76.2 $mL \cdot g_{VS}^{-1}$). On the other hand, the mono-digestion treatments of pig manure and sargassum reached 485.5 ± 19.8 $mL \cdot g_{VS}$ and 346.5 ± 57.1 $mL \cdot g_{VS}^{-1}$ respectively. The cumulative biogas yield of the sargassum mono-digestion (100S-0PM) was higher than the ones obtained by Paletta et al. (2023), whose study was the influence of Fe_2O_3 nanoparticles on the Anaerobic Digestion of *Sargassum* spp. finding that the addition of 10 $mg \cdot g_{VS}^{-1}$ Fe_2O_3 achieved the maximum total biogas yield of 101.9 ± 2.9 $mL \cdot g_{VS}^{-1}$.

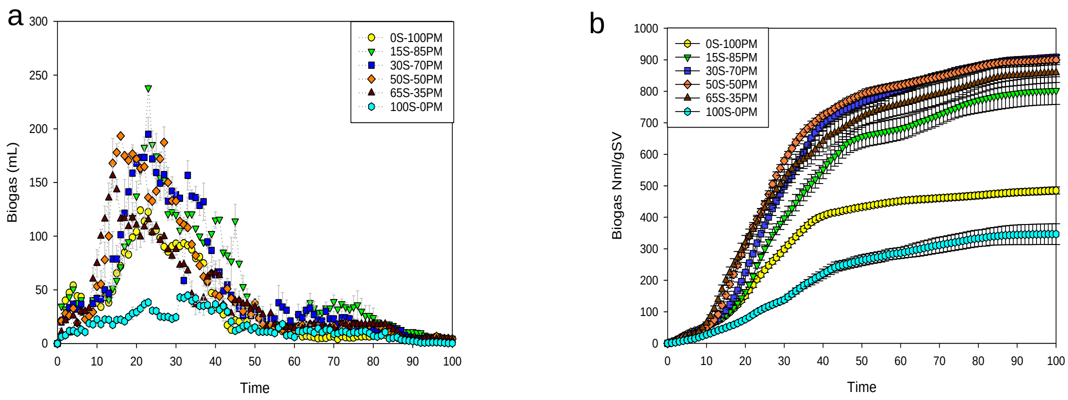

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Figure 3. Biogas production from the evaluated treatments.
a) Daily biogas production b) cumulative biogas yield. The data are the average of three replicates with standard errors.

3.3.3 Methane production and volatile fatty acids production

The profile of volatile fatty acids and the accumulated methane yield (AMY) as a function of time for each treatment can be seen in Fig. 4. The highest AMY was obtained with the treatment 30S-70PM (369.1 ± 6.9 mL $CH_4 \cdot g_{vsf}^{-1}$), followed by 50S-50PM (365.0 ± 7.2 mL $CH_4 \cdot g_{vsf}^{-1}$), 15S-85PM (321.4 ± 24.6 mL $CH_4 \cdot g_{vsf}$), 65S-35PM (319.6 ± 15.8 mL $CH_4 \cdot g_{vsf}^{-1}$) and finally the control treatments 0S-100PM (199.5 ± 4.8 mL $CH_4 \cdot g_{vsf}^{-1}$) and 100S-0PM (110.6 ± 1.3 mL $CH_4 \cdot g_{vsf}^{-1}$) . This indicates that the addition of 20 $mg \cdot L^{-1}$ Fe_3O_4 NPs promoted the production of methane on treatment 30S-70PM by 233.7 % and 85.01 % compared to the control of sargassum and pig manure, respectively.

Volatile fatty acids (VFAs), which include acetate, propionate, isobutirate, and butyrate, are important precursors of methane generation in AD (Thompson et al., 2021). The VFA profile presented in Fig. 4 showed a remarkable accumulation of acetate on the 3rd day of the experiment in co-digestion treatments including 15S-85PM, 30S-70PM, 50S-50PM, 65S-35PM. Registering average values of 3,476.2 $mg \bullet L^{-1}$, 3,483.3 $mg \bullet L^{-1}$, 2470.0 $mg \bullet L^{-1}$, 1866.0 $mg \bullet L^{-1}$ respectively. A subsequent diminution of acetate concentration took place between days 10 and 17 in all co-digestion treatments. It could happen due to the direct consumption of acetate by methanogens to produce CH_4 and CO_2 (Zhang et al., 2020). In the same period, an increase in biogas production was observed in the above-mentioned treatments (Fig. 2b). In the case of solo pig manure and sargassum, the acetate increment took more time (10-17 days).

The syntrophic interaction is the mutual thermodynamic dependence between bacteria and methanogens. It allows the other fermentation products to be converted into acetate, formate, or some other one-carbon compounds. (Schink, 1997). The hydrogenotrophic methanogens relation with hydrogen-producing acetogenic bacteria could explain the main pathway of the experiment. Due to the significant acetate consumption on the mentioned before treatments.

Furthermore, because of the addition of 20 $mg \cdot L^{-1}$ MNPs a DIET (Direct interspecies electron transfer) could be promoted. Due to the addition of conductive materials, DIET makes bacteria free from the traditional pathway of electron transfer (Zhang et al., 2023).

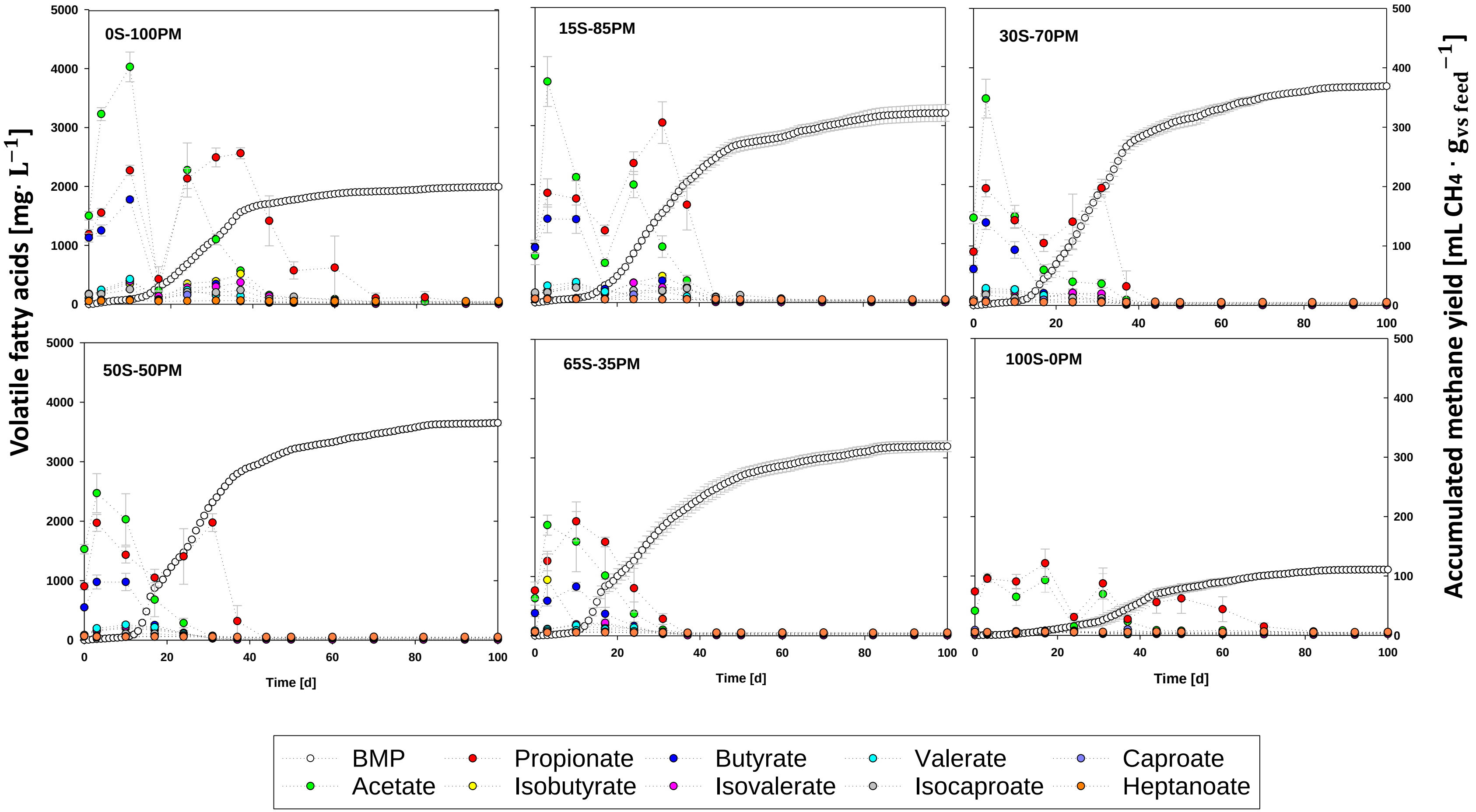

Figure 4. Accumulated methane yield and volatile fatty acids were obtained during the experiment.

A Principal Component Analysis (PCA) was applied to the most abundant VFA formed during the progress of anaerobic digestion. Acetate (A), propionate (P) and butyrate (B). The two components explained 82.3% of the variance.

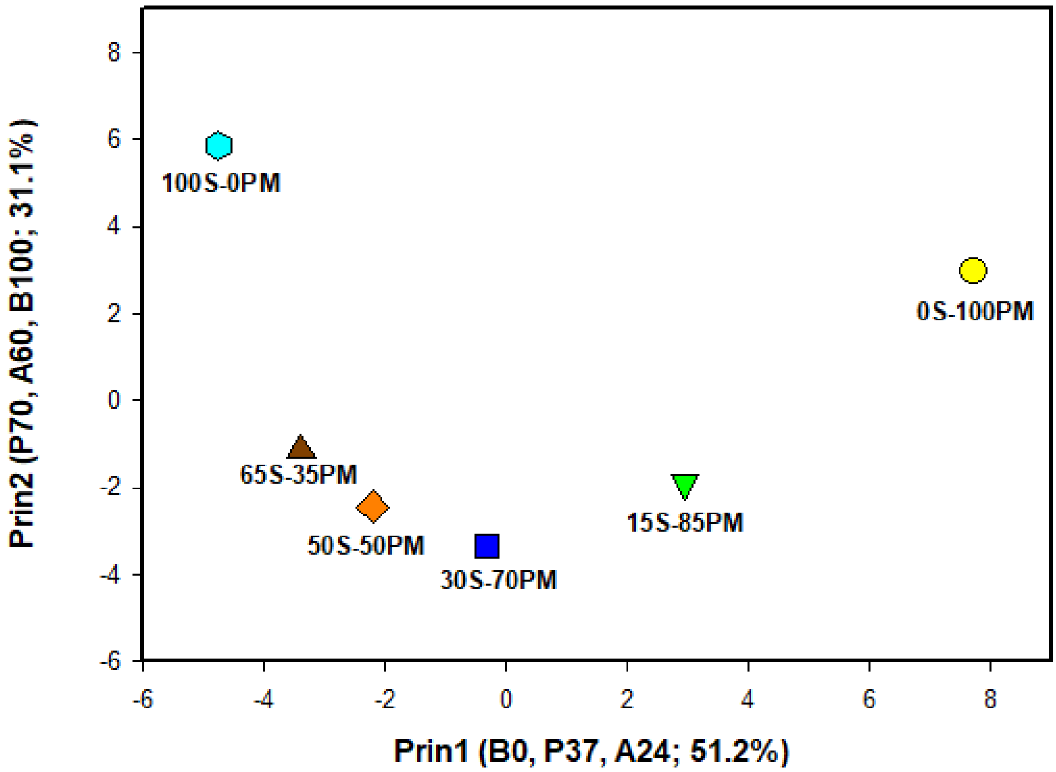

Figure 5. Principal Component Analysis based on VFAs profiles.

Solo pig manure 0S–100PM and 15S–85PM: Among the intermediate metabolites produced during the anaerobic digestion of organic wastes, propionate is one of the most important. Due to its easy accumulation, resulting in the acidification and eventual collapse of the anaerobic digestion system (Junrou Li et al., 2021). In the case of solo pig manure and 15S–85PM, there was an accumulation of acetate, propionate, and butyrate until day 10^{th} (Fig. 6). After that, there was a remarkable diminution on the 17^{th} day. It is important to highlight that the addition of magnetite nanoparticles could allow syntrophic cooperation between bacteria and methanogens (Zhang et al., 2020).

30S-70PM: This treatment shows its highest accumulation of acetate on the third day of the experiment, followed by a remarkable consumption on the 17th day. Propionate showed a final increment by 31st day. Butyrate remains stable after its unique peak on day 3rd (Fig. 6 d, e, f). This treatment obtained the highest accumulated methane yield. It could be due to its initial accumulation of acetate and almost immediate consumption.

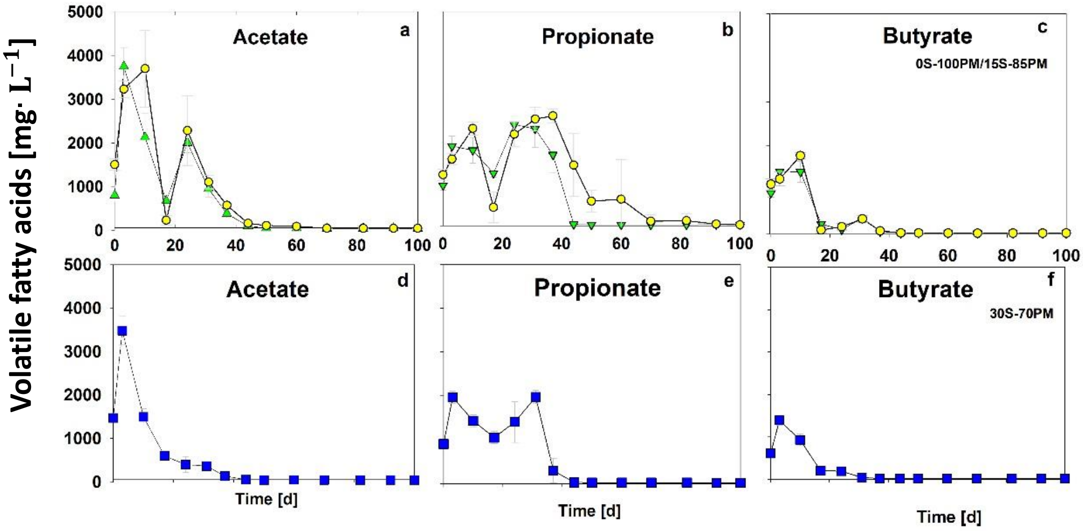

Figure 6. Degradation of VFAs for treatments 0S-100PM, 15S-85PM and 30S-70PM.

50S-50PM and 65S-35PM: In comparison with all other co-digestion treatments, these had the least VFA accumulation, due to their short adaptation phase (Fig. 7 a, b, c). An increase of buffer capacity of the system and therefore few effects of pH changes on anaerobic digestion could allow the VFA consumption (Lisboa & Lansing, 2013).

100S-0PM: This treatment obtained the lowest generation of VFAs (Fig. 7 c, d, f). It could be due to the low degradation of organic matter. This coincides with that reported by Thompson et al. (2021) and Rivera-Hernández et al. (2022), who found that raw sargassum exhibits lower concentrations of volatile fatty acids compared with the Hydrothermal pretreatment and co-digestion treatments used by researchers, respectively.

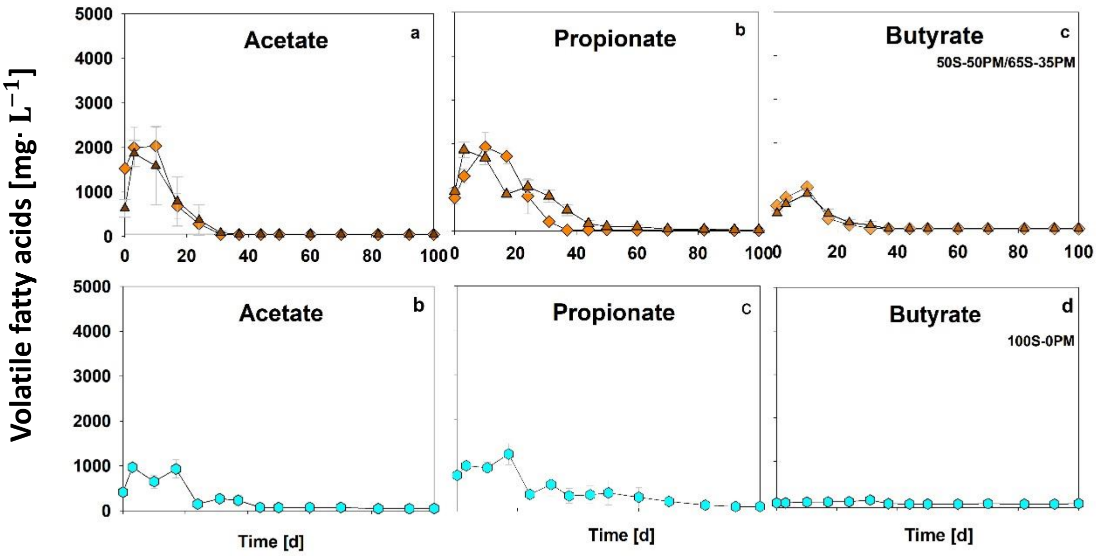

Figure 7. Degradation of VFAs for treatments 50S-50PM 65S-35PM and 100S-0PM.

The change in acetate, propionate and butyrate concentration with respect to time is presented on table 7.

Table 7. VFA declivity.

	VFAs Declivity	Acetate	Propionate	Butyrate
Treatments	0S-100PM	66.7	53.6	81.6
	15S-85PM	67.7	45.4	85.0
	30S-70PM	99.1	78.6	65.8
	50S-50PM	63.0	52.8	74.5
	65S-35PM	60.8	21.9	79.0
	100S-0PM	2.7	12.4	-

The highest declivity was on 30S-70PM treatment, in which the VFA degradation occurs between 3rd and 17th day. On the hand, the lower declivity was found on 100S-0PM. It could be due to substrate characteristics, which could cause the inhibition of the VFA production.

Table 8. Final Characterization of treatments.

Parameters		C: N	pH	TS (%)	VS/TS (%)	COD (mg·L^{-1})	Alkalinity (mg· L^{-1})	Sulfide ion (mg· L^{-1})	Sulfate ion (mg·L)
Treatments	**100 PM**	11	8.2	2.50 ± 0.3[a]	48.21 ± 10.2[a]	5,978 ± 17[a]	8,162 ± 202[a]	1,423 ± 26[a]	6,953 ± 150[a]
	15S-85PM	12.8	8.1	2.63 ± 0.1[ab]	56.73 ± 1.1 [a]	5,927 ± 91[ab]	7,854 ± 201[a]	1,274 ± 21[b]	6,895 ± 96[a]
	30S-70PM	14.5	8.1	2.83 ± 0.2[ab]	53.74 ± 7.0[a]	5,857 ± 76[ab]	7,205 ± 277[b]	1,378 ± 19[a]	6,463 ± 108[b]
	50S-50PM	16.8	8.8	2.41 ± 0.4[a]	42.27 ± 5.9 [ab]	5,796 ± 46[b]	6,281 ± 152[c]	610 ± 12[d]	5,198 ± 170[c]
	65S-35PM	18.5	8.6	2.55 ± 0.2[a]	41.75 ± 12.4 [ab]	5,453 ± 17[c]	5,247 ± 151[d]	804 ± 44[c]	5,359 ± 62[c]
	100 S	22.6	7.5	2.15 ± 0.1[a]	26.34 ± 3.7 [b]	3,927 ± 91[d]	3,740 ± 101 [e]	602 ± 37[d]	2,456 ± 76[d]

TS: total solids; VS: volatile solids; COD: chemical oxygen demand. The data are the average of three replicates with standard errors.
Means with the same letter in the same row do not present significant statistical difference, Tukey (*$p<0.05$*)

3.3.4 Modified Gompertz model

The modified Gompertz model parameters were evaluated for the individual replicates. By analyzing the changes and relations between each determination coefficient, it is possible to infer the effects of magnetite nanoparticle addition (Table 8). In terms of biochemical methane potential, no statistical difference ($p<0.05$) was observed among the highest values on 30S-70PM and 50S-50PM treatments. In contrast, the other treatments presented differences between each other.

In relation to the daily methane production rate, the addition of magnetite nanoparticles promoted the production by 11.7 and 11.8 mL $CH_4 \cdot g_{vsf}^{-1}$ in 30S-70PM and 50S-50PM, respectively. The mono-digestion of sargassum obtained the lowest methane production rate (2.5 mL $CH_4 \cdot g_{vsf}^{-1}$). It was no statistical differences between 0S-100PM, 15S-65PM and 65S-35PM treatments, their values ranged from 6.9 - 8.4 mL $CH_4 \cdot g_{vsf}^{-1}$.

In terms of lag phase, a time range was from 9.2 to 20.5 days, and no statistical difference ($p<0.05$) was observed among treatments 15S-85PM and 100S-0PM. The rest of the treatments were also grouped with no statistical differences.

Table 9. Modified Gompertz model parameters determined for each treatment.

Substrates	BMP [mL $CH_4 \cdot g_{vsf}^{-1}$]	μ_m [mL $CH_4 \cdot g_{vsf}^{-1}$]	λ [d]	R^2 [-]
0S-100PM D-EST	195.9^d ±4.2	6.9^b ±0.2	13.8^b ±1.1	0.998 0.997 0.998
15S-85PM D-EST	273.4^c ±15.9	8.1^b ±0.7	14.8^{ab} ±0.8	0.999 0.999 0.999
30S-70PM D-EST	361.0^a ±7.9	11.7^a ±1.4	14.2^b ±1.8	0.997 0.998 0.999
50S-50PM D-EST	355.7^a ±6.3	11.6^a ±0.4	11.0^b ±0.3	0.997 0.998 0.998
65S-35PM D-EST	315.2^b ±16.8	8.4^b ±1.0	9.2^b ±1.1	0.997 0.995 0.997
100S-0PM D-EST	112.6^e ±1.9	2.5^c ±0.2	20.5^a ±4.9	0.999 0.998 0.998

BMP: biochemical methane potential; μ: methane production rate, λ: lag phase time.
Means with the same letter within each column do not differ statistically. Tukey ($p<0.05$)

The enhancement of methane production under the experimental conditions compared with simulated conditions were 91.7% (65S-35PM), 136.6 % (50S-50PM), 156.9% (15S-85PM) and 168.2% (30S-70PM). It could be inferred that the co-digestion combined with the addition of magnetite nanoparticles had greater methane production than the sargassum and pig manure studied as separate substrates.

Table 10. Comparative studies about the effect of Fe_3O_4 nanoparticles in CH_4 production.

Fe_3O_4 concentration ($g \cdot L^{-1}$)	Substrate	Increase in CH_4 production (%)	Reference
1.50	Pure glycerol	16.0	(Im et al., 2019)
0.02	Animal manure	33.0	(Abdelwahab et al., 2022)
0.02	Chicken litter	73.9	(Aguilar-Moreno et al., 2020)
8.00	Pig manure	27.0	(Córdova et al., 2022)
0.20	Crude glycerol	49.8	(Boscaro et al., 2022)
0.10	Sargassum	26.97	(Paletta et al., 2023)
0.02	15S-85PM	156.9	This study
0.02	30S-70PM	168.2	This study
0.02	50S-50PM	136.6	This study
0.02	65S-35PM	91.7	This study

3.4 Conclusions

The synthesis of Fe_3O_4 NPs by the co-precipitation technique resulted in homogenous and small nanoparticles with an average diameter of 6.10 ± 1.14 nm. The addition of magnetite nanoparticles had a positive effect on biogas production, which was higher in the co-digestion treatments. Methane production was promoted by 233.7% and 85.01% on the 30S–70PM treatment compared to the control of sargassum and pig manure, respectively. Due to the significant acetate consumption identified in the co-digestion treatments, the hydrogenotrophic methanogens relation with hydrogen-producing acetogenic bacteria could explain the main pathway of the experiment.

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4. GENERAL CONCLUSIONS

The co-precipitation method employed for the synthesis of magnetite nanoparticles was effective, according to TEM and XRD analysis results.

The addition of magnetite nanoparticles promoted the biochemical methane potential in the co-digestion of 30S-70PM (361.0 mL $CH_4 \cdot g_{vsf}^{-1}$), follow by the treatment 50S-50PM with no statistical differences. Thus, if it is required to use more sargassum in the process, it is possible to choose the 50S-50PM treatment and obtain a positive result.

A considerable VFA degradation was similar in all co-digestion treatments and solo feed pig manure; differences between solo sargassum could be attributed to changes in methane production efficiency.

The use of magnetite NPs in the co-digestion of sargassum and pig manure could be valuable. Since there was better performance in the analyzed variables, however, it is necessary to explore different concentrations of Fe_3O_4 and evaluate the economic impact of their application.

5. ANNEXES

Annex 1. Declivity formula

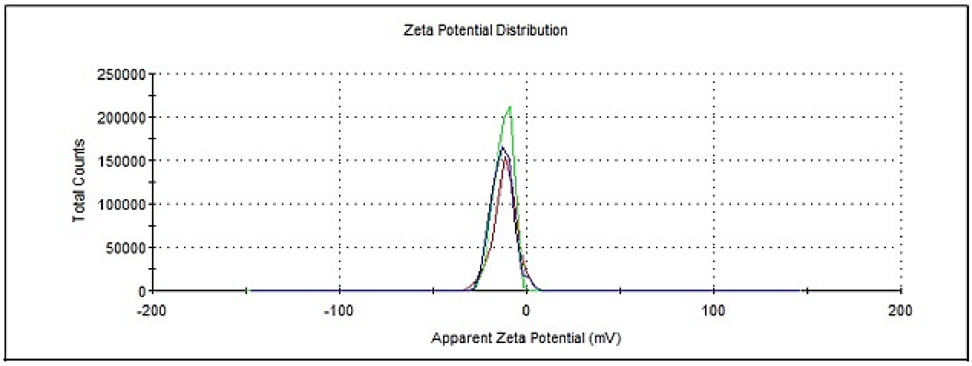
$$\text{Declivity} = \frac{\Delta y}{\Delta x} = \frac{\text{Change in VFA concentration}}{\text{Change in time}}$$

Annex 2. a) Hydrodynamic diameter and b) zeta potential of the synthesized nanoparticles.

a)

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-11.8	Peak 1:	-11.8	100.0	6.13
Zeta Deviation (mV):	6.13	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm):	0.182	Peak 3:	0.00	0.0	0.00

Result quality : Good



b)