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Effect of nickel and iron on hydrogen and methane production from organic solid waste in discontinuous processes

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PRESENTA:
M. en C. Karina Janette Salazar Batres

TUTOR PRINCIPAL:
Dr. Iván Moreno Andrade (Instituto de Ingeniería, UNAM)

MIEMBROS DEL COMITÉ TUTOR:
Dr. Alfonso Durán Moreno (Facultad de Química, UNAM)
Dr. Julián Carrillo Reyes (Instituto de Ingeniería, UNAM)

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JURADO ASIGNADO:

Presidente:	Dra. Rosalinda Campuzano Ángeles
Secretario:	Dr. Alfonso Durán Moreno
1 er. Vocal:	Dr. Philippe Constant
2 do. Vocal:	Dr. Julián Carrillo Reyes
3 er. Vocal:	Dr. Iván Moreno Andrade

Lugar donde se realizó la tesis: Laboratorio de Procesos Avanzado en tratamiento de aguas, Instituto de Ingeniería, UNAM, campus Juriquilla, Querétaro.

DIRECTOR DE TESIS:

Iván Moreno Andrade

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AD	Anaerobic digestion
AE	Acidogenic effluents
AMPTS II	Automatic methane potential test
ANCOM-BC 2	Analysis of compositions of microbiomes with bias correction
BMP	Biochemical methane potential assay
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
dbRDA	distance-based redundancy analysis
DF	Dark fermentation
FDH	Enzyme formate dehydrogenase
FW	Food waste
HRT	Hydraulic retention time
HY	Hydrogen yield
MCFA	Medium-chain fatty acids
MCR	Methyl coenzyme M reductase
NP	Nanoparticles
OFMSW	Organic fraction of municipal solid waste
OLR	Organic loading rate
OSAD	One stage anaerobic digestion
OFMSW	Organic solid waste
PFL	Pyruvate-formate lyase
PFOR	Pyruvate-ferredoxin oxidoreductase
PLC	Programmable logic controller
rpm	Revolutions per minute
S/I	Substrate/inoculum ratio
SBR	Sequencing Batch Reactor
SMY	Specific methane yield
TM	Trace metals
TS	Total solids
TSAD	Two-stage anaerobic digestion
UASB	Upflow anaerobic sludge bed
VFA	Volatile fatty acids
VS	Volatile solids
$VS_{inoculum}$	Volatile solids of inoculum

1 ABSTRACT

In recent years, alternative renewable energy generation sources have been investigated, highlighting dark fermentation and anaerobic digestion processes due to their potential to obtain hydrogen-rich and methane-rich biogas. Different trace metals (TM) intervene in these biological processes. Two of the most important TM are Fe^{2+} and Ni^{2+} since they can improve process stability. These metals are part of cofactors of enzymes and microorganisms' growth. This research was focused on determining the effect of the supplementation of different concentrations of TM on two-stage anaerobic digestion (TSAD) compared with one-stage anaerobic digestion (OSAD). The results showed that it was possible to increase the CH_4-productivity and the SMY by 72% and 105%, respectively, by operating in TSAD compared with OSAD. The Ni^{2+} addition improved the stability of the first and second stages, allowing higher biogas production. The microbial communities' composition at phylum level changed in each stage. Regarding Fe^{2+} and Ni^{2+} addition, the TM concentrations that increased the hydrogen yield in batch were 0.25 mg/L of Ni^{2+} and 334 mg/L of Fe^{2+}. In the case of the methanogenic reactor, the undiluted AE without TM caused the fast decay of the process. The AE enriched with TM increased the specific methane yield and avoided inhibition. The results showed that when using AE enriched with Fe^{2+} and Ni^{2+} for CH_4-rich biogas production, it was possible to operate stably considering the variations in the OLR due to the concentration of AE.

2 INTRODUCTION AND LITERATURE REVIEW

Anaerobic digestion (AD) is a biological conversion process without an external electron acceptor, such as oxygen (Angelidaki and Sanders, 2004; Metcalf & Eddy, 2003). It involves sequential and parallel steps known as hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Choong *et al*., 2016; Liu *et al*., 2019). Various wastes have been used as substrates to generate methane-rich biogas, which may be transformed into electrical or thermal energy; thus, an enhancement of efficiency in methane generation from different substrates is proportional to more energy recovery (Nevzorova and Kutcherov, 2019).

Some AD-related issues, such as process instability and low methane yield, limit this technique to a generalized application (Chen *et a*l., 2008). Due to the inadequacy of energy recovery from waste, strategies have been used to increase the biogas yield and achieve more energy production (Li *et al*., 2019), where the administration of trace metals (TM) is one of the most favorable (Jarvis *et al*., 1997; Moestedt *et al*., 2016). Besides the operational parameters in the digester, biogas yield is also determined by the content of macroelements (Bożym *et al*., 2015; Lar *et al*., 2010) and trace elements of which several are metals (Pobeheim *et al*., 2010; Zandvoort *et al*., 2006) such as Fe, Co and Ni that are part of cofactors of enzymes implicated in the synthesis of methane and the growth of microorganisms (Bougrier *et al*., 2018). Methanogenesis is considered one of the most metal-rich enzymatic pathways; iron is the most abundant metal, followed by nickel, cobalt and smaller quantities of zinc and molybdenum (Hijazi *et al*., 2020). However, TM effects highly depend on the supplemental concentrations (Wanli Zhang *et al*., 2015), which are generally added in excessive amounts, and the above may result in inhibitory effects on the processes (Thanh *et al*., 2016). For this reason, it is necessary to carry out more research focusing on determining the frequency of TM addition, according to indicators such as the accumulation of volatile fatty acids or low methane yields.

Research related to TM has been focused on the stages of acetogenesis and methanogenesis, therefore, it is important to explore the TM influence in the stages of hydrolysis and acidogenesis since the system requirements at each stage may differ. In a two stages system, it is possible to obtain a H_2 and CH_4-rich biogas, besides, removal efficiencies of organic matter could increase, the stability of pH and alkalinity improves as well as the C/N ratio (Paudel *et al*., 2017). With the TM addition in the first stages, higher stability could be achieved, which implies a higher biogas production. Acidogenic effluents (AE) enriched with TM could improve the second stage, allowing the use of high organic loading rates.

2.1 Organic fraction of municipal solid waste (OFMSW)

About 2.01 billion tons of municipal solid waste are generated annually. It is estimated that the generation per capita is 0.74 kg; however, the range is broad since it goes from 0.11 to 4.54 kilograms; within the composition of waste generated in high-income countries, food, and green waste account for 32%, while middle and low-income countries generate 53% and 56%, respectively. The composition of urban solid waste may vary depending on the consumption patterns of its population; however, as levels of economic development decline, the fraction of organic waste increases (Kaza *et al*., 2018). The OFMSW mainly refers to a mixture of food waste, leaf, and yard waste (Cesaro, 2021). The OFMSW composition depends on the place and time of collection for a specific municipality or area, number of inhabitants, their social condition, predominant economic activities, regional food habits, and season (Alibardi and Cossu, 2015a; Campuzano and González-Martínez, 2016), nevertheless, it usually contains high lignocellulosic and fatty fractions due to a large content of food waste, kitchen waste and leftovers from residences, cafeterias, and markets (Shah *et al*., 2016).

A simulated OFMSW has been prepared to be used as a substrate in biohydrogen production. Alavi-Borazjani *et al*., (2021), elaborated a complex OFMSW that contained 95% of food waste (fruit and vegetables, cooked pasta and rice, cooked meat and fish, bread and bakery, cheese, and biscuits) and 5% paper. Similarly, Yeshanew *et al*., (2018) elaborated a substrate that contained 7% beef meat, 3.9% coffee,

4.3% rice, 20.9% potatoes, 5.1% bread, 5.1% garden waste, 1.9% yogurt, 31.7% white office paper, 16.7% packing cardboard, and 3.4% color cardboard; the composition of these substrates is similar to the OFMSW described in studies where the sample was taken directly from a waste separation plant (Alibardi and Cossu, 2015a; Cesaro, 2021; Ebrahimian and Karimi, 2020; Shah *et al.*, 2016). However, in other studies, OFMSW was simulated using food waste from cafeterias or restaurants, and it was mixed with paper. Escamilla-Alvarado *et al*., (2015, 2013) performed the mixture de food waste and office paper at 60:40 ratio. Gómez *et al.*, (2006) and Redondas *et al*., (2015) prepared a substrate with 10% banana, 10% apple, 10% orange, 35% cabbage, 25% potatoes, 8% bread, and 2% paper. Muñoz-Páez *et al.*, (2012) and Valdez-Vazquez *et al*., (2006) prepared a substrate that contained 40% of paper and 60% food waste. The last examples correspond to mixtures with a smaller variety of residues. According to Alibardi and Cossu, (2015a), the different origins and compositions of the organic waste samples, coupled with different process conditions, might affect the high variability of hydrogen production yields. The high variation in the OFMSW characteristic result in differences in the H_2 production.

Physicochemical characterization of different samples of OFMSW may vary according to its composition since it has been reported that the TS content ranges from 19.3 to 75%. The OFMSW samples with a higher percentage of moisture are those that contain more fruits and vegetable waste; whereas, when it contains more than 40% of paper and cardboard waste, the moisture is lower, as in the case of the OFMSW used by Paillet *et al*., (2021, 2020), which was reconstructed according to the characteristics of the OFMSW collected in France. The carbohydrate concentration in OFMSW varies, as reported by various authors as Sharma and Melkania, (2018a, 2018b, 2018c), who indicate that the OFMSW samples from a municipal landfill located in Uttarakhand, India, presented different carbohydrate contents ranged from 31.4±2.42 to 48.6±5.21 g/L even though the sample was collected from the same location. Carbohydrates content is crucial since it has been found that there is a linear correlation between its content and the production of hydrogen-rich biogas; also, the chemical composition of the substrates has an influence on the final products of dark fermentation (Alibardi and Cossu, 2016a).

2.1.1 Potential of OFMSW to biohydrogen production

Dark Fermentation has been proposed as a promising technique to produce clean hydrogen due to its low chemical energy requirement and, therefore, more environmentally friendly compared to the conventional chemical process (Jarunglumlert *et al*., 2018). This biological process is divided into two stages which are hydrolysis and acidogenesis (Fig. 1). During hydrolysis, complex organic polymers are hydrolysed into simple soluble organic compounds; subsequently, the generation of volatile fatty acids, H_2, CO_2, and other intermediates occurs during the acidogenesis (Angeriz-Campoy *et al.*, 2018). H_2 is the cleanest carbon-free fuel with the highest energy content (120 MJ/kg) compared to methane (50 MJ/kg), gasoline (44 MJ/kg), and ethanol (26.8 MJ/kg) (Ebrahimian and Karimi, 2020); however, worldwide hydrogen production mainly comes from fossil fuel technologies (>95%) and only 1% of H_2 is produced from biomass (Dauptain *et al.*, 2021). Hence, hydrogen production by the process of dark fermentation using OFMSW has a promising potential for biofuels generation and high-value products (Ebrahimian and Karimi, 2020; Kobayashi *et al.*, 2012) since there are numerous studies where relatively high yields have been achieved, as shown in Table 1.

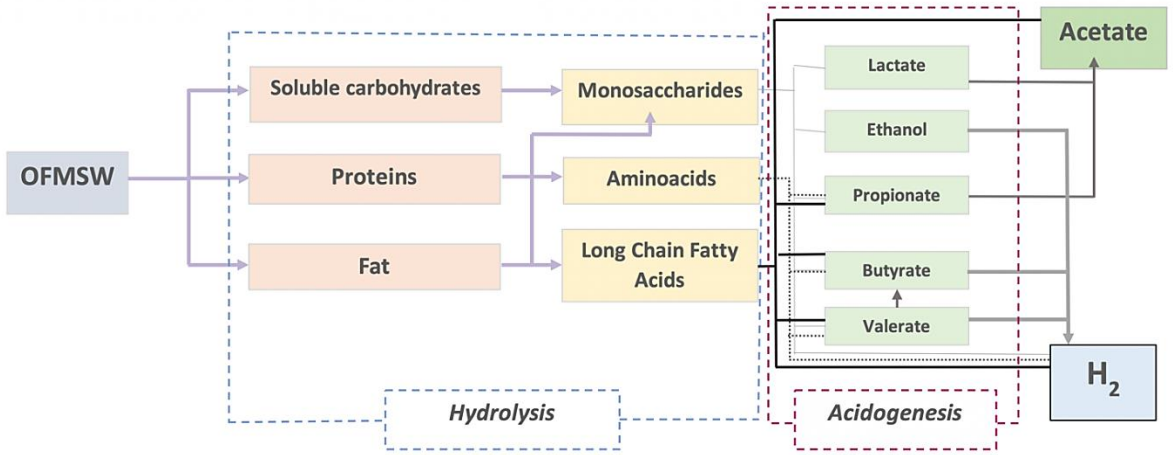


Fig. 1. Dark fermentation steps and microbiological pathways (from Salazar-Batres *et al*., (2022)).

Bio-hydrogen yields and productivities reported vary widely due to different operational parameters used in each experiment, such as hydraulic retention time (HRT), substrate concentration, organic loading rate (OLR), temperature, and pH (Castelló *et al*., 2020). The optimum pH is specific to each type of substrate (Ziara *et al*., 2019). According to Baldi *et al.*, (2019b), it is possible to increase the hydrogen production if the pH value is maintained between 5 and 6.5 since the metabolic pathways of acetate and butyrate predominate under these conditions. On the other hand, strongly acidic or basic pHs negatively affect the activity of hydrogen-producing bacteria since ATP would be used to ensure cell neutrality rather than to produce hydrogen. Additionally, hydrogen production could be affected by pH values less than five since hydrogenase activity could be inhibited. Moreover, low pH values are favorable for *Clostridium* sp. to produce solvents such as ethanol, butanol, and acetone rather than hydrogen (Akhlaghi and Najafpour-Darzi, 2020).

Due to the possibility of keeping the dark fermentation process stable operating at pH close to 5.5, numerous experiments have been carried out under these conditions (Bru *et al*., 2012; Cuetos *et al*., 2007; Gómez *et al*., 2006; Lavagnolo *et al.*, 2018; Redondas *et al*., 2015; Sharma and Melkania, 2018b, 2018c; Tyagi et al., 2014). Favaro *et al*., (2013), proved that when batch reactors are operated at a pH of 5.5, a higher hydrogen yield can be obtained (70.1±4.1 mLH_2/gVS) against a yield of 23.4 ± 2.9 mLH_2/gVS when operated at a pH of 7. Baldi *et al*., (2019b) achieved better control over the fermentation process in terms of kinetics and pH stability when using an automatic pH control strategy since adding a buffer solution at the beginning of the experiment was not enough to maintain adequate control. They obtained the highest yields in the same study with maximum average values ranging from 68.5 and 88.5 LH_2/KgTVS operating at pH of 5.5 and 6.5. However, Kvesitadze *et al*., (2012), carried out the operation of a batch reactor with a pH of 9, being possible to inhibit the methanogenic activity. It was noticed that the percentage of hydrogen in bioH_2 produced at initial pH 9.0 and pH 5.5 was almost equal. Nonetheless, a significant difference was noted in cumulative production of gas, being 3.5 times higher in operation with a pH of 9 than the one with a pH of 5.5.

Table 1. Biohydrogen yield on different reactor types using organic fraction municipal solid waste (OFMSW) as substrate (from Salazar-Batres *et al*., (2022)).

Reactor type *	Conditions**	pH	%H_2 in gas	H_2 Yield	Reference
Batch	I/S ratio: 0.5, T 55°C	7.0		62.5 $LH_2/kgVS_{added}$	(Alavi-Borazjani *et al*., 2021a)
Batch	T 34 °C		22±0.9	30 $LH_2/kgVS_{added}$	(Redondas *et al.*, 2015)
Batch	T 35 ± 1°C	7.5		85 ± 3 BHP $LH_2/kgVS$	(Alibardi and Cossu, 2015a)

Batch	I/S ratio: 5% w/w. OLR 16 VS/kg/d, HRT 24 h, T 38 ± 2°C		47	99 mL H_2/$gVS_{removed}$	(Alzate-Gaviria *et al.*, 2007)
Batch	T 38.0°C ± 1.0°C	6.5		104.5±0.7 LH_2/kgTVS	(Baldi *et al.*, 2019b)
Batch	I/S ratio: 10 gVS substrate/gVS inoculum, T 37±1 °C	6.0		31.6 LH_2/kgVS	(Cesaro *et al*., 2020)
Batch	S_0/X_0 20±5 (gVS/gVS), T 37 °C	6.0		40±3 LH_2/$kgVS_{added}$	(Dauptain *et al.*, 2021)
Batch	Stirred manually twice a day, T 37 °C	5.5-8.9		119.7 LH_2/kgVS	(Dong *et al.*, 2010)
Batch	Stirred at 160 rpm, T 37°C	6.8		151 LH_2/kg of substrate	(Ebrahimian and Karimi, 2020)
Batch	I/S ratio: 1 gVS/gVS, T 35±2°C	5.5		70.1 ± 4.1 LH_2/kgVS	(Favaro *et al*., 2013)
Batch	F/M=6, T 35±1 °C	5.5		29.8 LH_2/kgVS	(Lavagnolo *et al.*, 2018)
Batch	S/I ratio 10, T 37 °C	6.0		40.8 ± 0.5 LH_2/kgVS	(Paillet *et al.*, 2020)
Batch	HRT 60 h, T 30 °C	7.9	46.7	246.93 LH_2/kgTVS	(Sekoai and Kana, 2014)
Batch	T 37°C	7.0	39	61 L H_2/$kgVS_{added}$	(Shah *et al*., 2016)
Batch	T 37°C	5.5	67.9	43.68 LH_2/kgCarbo	(Sharma and Melkania, 2018a)
Batch	T 55°C	5.5	36%	51 LH_2/$kgVS_{removed}$	(Tyagi *et al*., 2014)
Batch	The substrate to inoculum ratio (S/I) was 20 $gVS_{substrate}$/$gVS_{inoculum}$, T 35±2°C			41.7±2.3 mL H_2/gVS_{added}	(Yeshanew *et al*., 2018)
Batch	Stirred at 50 rpm, T 55°C	9.0	50%	82.5 LH_2/kgVS	(Kvesitadze *et al.*, 2012)
UASB	the UASB was filled with the inocula suspension (2 g/L) and sucrose (3.85 L), HRT 24 h, T 38±2°C	5.7	51%	127 LH_2/$kgVS_{removed}$	(Alzate-Gaviria *et al.*, 2007)
CSTR	OLR 36 ± 2 gVS L/L d, HRT 3 d, Stirred at 100 rpm, T 55 ± 1°C	5.7	35 ± 4%	60±4 LH_2/$kgVS_{added}$	(Tenca *et al.*, 2011)
CSTR	OLR 16 kg TVS/m3 d, HRT 3d, T 55°C			29±5 LH_2/$kgTVS_{added}$	(Zahedi *et al*., 2016)
Semi-continuous	OLR 75.6 (g TVS/L/d) HRT 1.9 d, Stirred at 12 rpm, T 55±0.5 °C		51.0±1.5	50.9 LH_2/$kgVS_{added}$	(Angeriz-Campoy et al., 2017)
Semi-continuous	HRT 1.2 d, T 55°C	5.5	52.4	33.8 LH_2/$kgVS_{added}$	(Angeriz-Campoy *et al.*, 2018)
Semi-continuous	OLR 66 gTVS/L/d, Stirred at 12 rpm, T 55°C	5.5	44	38 LH_2/$kgVS_{added}$	(Angeriz-Campoy *et al.*, 2015)
Semi-continuous	T 34°C	5.0-6.0	24.7	N.A.	(Cuetos *et al.*, 2007)
Semi-continuous	OLR 8.6 gSV/kg/d, T 55°C	6.3		123 mLH_2/kg/d	(Escamilla-Alvarado *et al.*, 2013)

* continuous stirred tank reactor (CSTR), Upflow anaerobic sludge bed (UASB),

**Inoculum/substrate ratio (I/S),Temperature (T), Initial Substrate to Microorganisms ratio (S_0/X_0), Hydraulic Retention Time (HRT).

Hydrogen production from OFMSW may also be influenced by temperature; the operational ranges are mesophilic (25-40 °C), thermophilic (40-65 °C), extreme thermophilic (65-80 °C), or hyperthermophilic (>80 °C) (Gopalakrishnan *et al*., 2019). The structure of the bacterial community and the metabolic pathways are affected by varying the temperature (Toledo-Alarcón *et al*., 2018). Most of the experiments on this subject have been carried out under mesophilic conditions (Alibardi and Cossu, 2015a; Alzate-Gaviria *et al*., 2007; Baldi *et al*., 2019b; Bru *et al*., 2012; Cesaro *et al*., 2020; Cuetos *et al*., 2007; Dauptain *et al.*, 2021; Dong *et al*., 2010; Ebrahimian and Karimi, 2020; Favaro *et al.*, 2013; Gómez *et al*., 2006; Lavagnolo *et al.*, 2018; Lay *et al.*, 1999; Paillet *et al*., 2021; Redondas *et al*., 2015; Sekoai and Kana, 2014; Shah *et al.*, 2016; Sharma and Melkania, 2018b, 2018c, 2018a; Yeshanew *et al.*, 2018) with a prevailing range of 35 to 37 ° C. On the other hand, in the experiments carried out under thermophilic conditions, the temperature of 55 ° C predominates (Alavi-Borazjani *et al.*, 2021a; Angeriz-Campoy *et al.*, 2015, 2017, 2018; Escamilla-Alvarado *et al.*, 2013; Kumar Tyagi *et al.*, 2014; Kvesitadze *et al*., 2012; Tenca *et al.*, 2011; Tyagi *et al*., 2014; Zahedi *et al.*, 2016).

Valdez-Vazquez *et al.*, (2005), evaluated the effect of temperature in the mesophilic and thermophilic regime of semicontinuous, acidogenic solid substrate anaerobic digestion of the OFMSW, obtaining as a result that under thermophilic conditions, it is possible to obtain a higher percentage of hydrogen 58% and 42%, respectively and a higher yield 360 N mL H_2/g $VS_{removed}$ than those obtained in 42% mesophilic

conditions and 165 N mL H_2/g $VS_{removed}$. In general, under thermophilic conditions, several benefits are obtained, such as higher process rates, better sanitation, higher degradation of persistent organics, higher bioavailability of absorbed compounds for degradation, and higher solubility of hydrophobic compounds (Tyagi *et al*., 2018). However, extreme thermophilic conditions may not be self-supported due to intensive-energy requirements to maintain the high temperatures (Gopalakrishnan *et al.,* 2019).

Another critical factor to consider besides the operational parameters is the inoculum since influenced greatly the hydrogen yields and final microbial communities. Therefore, it is necessary to add it to start the hydrogen production process (mainly for the evaluation of H_2 potential test in batch process), whose primary requirement for an efficient H_2 production process is linked to the availability of mixed microbial consortia in which H_2-consuming and non-hydrogen producing bacteria are suppressed (Dauptain *et al.*, 2021; Favaro *et al.,* 2013). To increase H_2 production through proper inoculum selection, it is necessary to remove hydrogen-consuming and non-hydrogen-producing bacteria from mixed microbial consortia; for this, several pretreatment methods have been proposed, including heat treatment acidification, basification, aeration, or freezing (Favaro *et al.,* 2013). Heat shock pretreatment predominates among the pretreatments used in hydrogen production from OFMSW since it is possible to gather hydrogen producers and inactivate methanogens (Akhlaghi *et al.,* 2019a).

2.1.2 Potential of OFMSW to methane production

Biogas produced through AD of renewable feedstocks such as OFMSW is one of the most promising alternatives to fossil-derived energy. AD is suitable for converting diverse and complex feedstocks into methane-rich biogas (Sawatdeenarunat *et al*., 2015). Methane production from OFMSW can be obtained under wet (<10% total solids) or solid (>20% total solids), mesophilic (35–40 °C) or thermophilic (>55 °C), batch or continuous, and single or two stage systems (Zeshan *et al*., 2012). Table 2 summarizes the yields obtained during the production of methane-rich biogas from OFMSW of various origins.

Table 2. Literature review of potential of OFMSW to methane production.

Reactor type	Feedstock	Conditions	CH_4 Yield (mL CH_4/gVS)	Reference
Batch	OFMSW	35 °C, reaction 30 d	196	(Jojoa-Unigarro and González-Martínez, 2023)
SBR	Food waste from a market	37 °C, HRT 10.4 d, OLR 10 gVS/L·d	275	(Jiménez-Ocampo et al., 2021)
Semicontinuous	Food waste from a canteen	37 °C, HRT 20 d, OLR 5.5 gVS/L·d	184	(Ma et al., 2020)
Semicontinuous	Food waste sorted from OFMSW	HRT 17 d, 35 °C, OLR 2.5 kgVS/m^3 ·d	694	(Baldi et al., 2019a)
Semicontinuous	Food waste from a canteen	35 °C, OLR 2.4 gVS/L ·d, HRT 25 d	437	(Zhang et al., 2019)
CSTR	Fruit and vegetable waste and food waste	HRT 30 d, 37 °C, OLR 4.0 gVS/L·d	328	(Shen et al., 2013)

The yields obtained vary according to the operating conditions, pH, temperature, OLR, and HRT. The composition of the feedstock is another parameter that has a direct impact on the recovery of biogas rich in methane. The OFMSW feedstock quality is assessed based on organic matter separation, solubilization, and biodegradability condition (Cesaro and Belgiorno, 2014).

2.2 Biohydrogen production (Dark fermentation)

Biological hydrogen production is a promising alternative for the production of fuel from low-cost, renewable, and environmentally friendly resources (Akhlaghi and Najafpour-Darzi, 2020), such as the OFMSW, since it represents several benefits that combine waste minimization, energy recovery, and valorization (Yeshanew *et al*., 2018); it provides an ecological solution for managing organic waste since it is possible to convert this waste into biofuel (Sharma and Melkania, 2018c). The high calorific power of OFMSW makes it a suitable substrate for bioenergy generation (Paillet *et al*., 2021) due to its rich content of carbohydrates, biodegradability (Sekoai and Kana, 2014), and also because it is all year available and apparently free of cost (Escamilla-Alvarado *et al.*, 2015).

2.2.1 Microorganisms in biohydrogen production from OFMSW

Microorganisms in dark fermentation process belongs to obligate or facultative anaerobes (Mathews and Wang, 2009), with particular metabolic pathways in which different types of enzymes and coenzymes are involved, leading to differential yields of H_2. The substrate composition influences the microbial composition and determine the dominance of some group of microorganisms (Zahedi *et al*., 2014). The H_2 producing bacteria can be divided into three groups: spore-forming obligate anaerobes (e.g., *Clostridium* spp.), non-spore-forming obligate anaerobes (belong to the phylum Firmicutes and Bacteroidetes), and facultative anaerobes with fermentative metabolism (e.g., members of the Enterobacteriaceae and Bacillaceae families, among which genera such *as Enterobacter* and *Bacillus*, respectively (Cabrol *et al*., 2017).

The heterogeneous composition of the OFMSW as substrate promote the growth of a complex community in dark fermentation. A positive interaction can be obtained from microorganisms that are not capable to produce H_2 or with low-efficient H_2 producers, including the regulation of O_2 presence (e.g., *Bacillus*, *Klebsiella*), the acidification of the media (e.g., lactic acid bacteria), and the oxidation of short chain fatty acids, preventing their accumulation and achieving a buffering effect (e.g., *Leuconostocaeae*, *Streptococcaceae)* (Cabrol *et al*., 2017).

In the particular case of organic solid waste reactors for H_2 production, some non-usual H_2 producers has been reported as dominant species including some consumers of lactic acids as *Megasphaera* or propionic acid as *Syntrophobacter, Syntrophomonas* (Moreno-Andrade *et al.*, 2015), of microorganisms that produce formation of granules (as *Prevotella, Klebsiella),* preventing the washing or loss of biomass and offering barriers against toxic or hostile environments for hydrogen producing bacterias (Cabrol *et al.*, 2017; Liang *et al*., 2010).

The majority of microbial studies for H_2 production has been wide studied in wastewater and simple or synthetic substrates (e.g., glucose) resulting in the mainly dominance of *Clostridium* spp. However, the reports for OFMSW reflect other dominance genera associated to the complexity of the substrate. Elsamadony *et al.* (2015), reported a microbial consortium with 92–93% affiliated to *Enterobacter*, *Escherichia*, *Buttiauxella*, and *Pantoea* as the H_2-producing bacteria. *Bacillus* sp. has been also demonstrated their ability to convert OFMSW into H_2 (Shah et al., 2016).

In case of high solid process phylogenetic analysis of samples revealed the dominance of *Pseudomonas fulva* with similarity of 99% (Elsamadony *et al.*, 2015). The presence/dominance of facultative anaerobes (e.g. Enterobacteriaceae, *Bacillus*, *Shewanella*, *Pseudomonas*), alone or in combination, have been reported to be preferable under real variable and unsterile conditions, and enabled to reach similar or higher H_2 yields than the conventional process with *Clostridium* spp. dominance, especially under very specific operating conditions such as recalcitrant substrate (Cabrol *et al.*, 2017).

The microbial dynamic change as the reactors are acclimated, reducing the microbial diversity. Paillet *et al.*, (2021) reported the reduction of abundance of microorganisms from the beginning of reactor operation

using OFMSW (Pseudomonadales, Clostridiales, Lactobacillales and Bacillales) shift of the microbial community with the dominance of Clostridiales and Lactobacillales (relative abundances ranging from 37 to 63% and from 5% to 60%, respectively) showing good stability on the microbial community. Concerning the Lactobacillales order, all the species observed were related to the genus *Lactobacillus* (five species obtained, described all as lactate producers). The equilibrium observed between Lactobacillales and Clostridiales can be related to the stable H_2 production, since species as *C. beijerinckii* and *C. butyricum* are able to consume lactate and acetate to produce butyrate and hydrogen (García-Depraect *et al.*, 2019). The role of lactic acid bacteria on the H_2 production, specially from OFMSW, need to be studied in order to develop an efficient process.

2.2.2 Effect of trace elements (TE) on bio-hydrogen production

To improve the capacity of a biogas plant and create more favorable conditions for the microorganisms present in the bioreactor, adequate availability of micro and macronutrients are required, mainly when single substrates rather than complex mixtures of materials are used. Besides essential macronutrients such as carbon (C), nitrogen (N), phosphorus (P), and Sulphur (S), the anaerobes also require the growing factor of trace elements at relatively lower concentrations (Choong *et al.*, 2016; Nordell *et al*., 2016; Pobeheim *et al*., 2010). A trace element is a chemical element whose concentration is very low, these elements are essential components of cofactors and enzymes (Hijazi *et al*., 2020; Thanh *et al.*, 2016).

Trace elements play an essential role in the metabolic physiology and proliferation of bacteria producing hydrogen during fermentation. The enzymes that catalyze the hydrogen production reactions are classified as [Fe-Fe]–hydrogenases, [Ni-Fe]-hydrogenases, and [Ni–Fe–Se]-hydrogenases. The [Ni-Fe]-hydrogenase consist of a [Ni-Fe]-active site and three FeS clusters. In turn, the active sites of [Fe-Fe]-hydrogenase include an Fe_2S_2 core connected to an Fe_4S_4 cluster acting as a ligand. Hydrogenases capture electrons from reduced ferredoxin, resulting in the formation of molecular hydrogen. The primary function of [Fe-Ni] active site is reducing protons using electrons transfer by Fe-S clusters. [Fe-Ni] Hydrogenases are efficient biological catalysts, which can interconvert protons, electrons, and molecular hydrogen (Chen *et al*., 2021; Cieciura-Włoch *et al*., 2020).

Biohydrogen is produced by two different pathways where carbohydrates can be consumed, producing pyruvate, which is later converted to acetyl coenzyme A. Both pyruvate and acetyl coenzyme A can be reduced by pyruvate-ferredoxin oxidoreductase (PFOR) or pyruvate-formate lyase (PFL). Microorganisms are limited to produce only 2 mol of biohydrogen once they are driven by PFL pathway because they are not able to assimilate NADH for the formation of more biohydrogen. NADH may be oxidized if microorganisms take pathway PFOR, since this metabolic route allows [FeFe]- hydrogenases not only to promote this oxidization but also accelerate this process. Basically, biohydrogen production is carried through pyruvate-ferredoxin oxidoreductase (PFOR) and in small amounts through NADH-ferredoxin oxidoreductase (NFOR). These enzymatic complexes transfer electrons to [FeFe]-hydrogenase, which is responsible for biohydrogen production by a reversible reduction of protons accumulated throughout fermentation (do Nascimento Junior *et al.*, 2021). According to Constant and Hallenbeck, (2019), there are various possibilities for the connection of different hydrogenases in Clostridia to glycolysis and pyruvate degradation. Fig. 2 shows [FeFe]-hydrogenases and the pyruvate junction of metabolism.

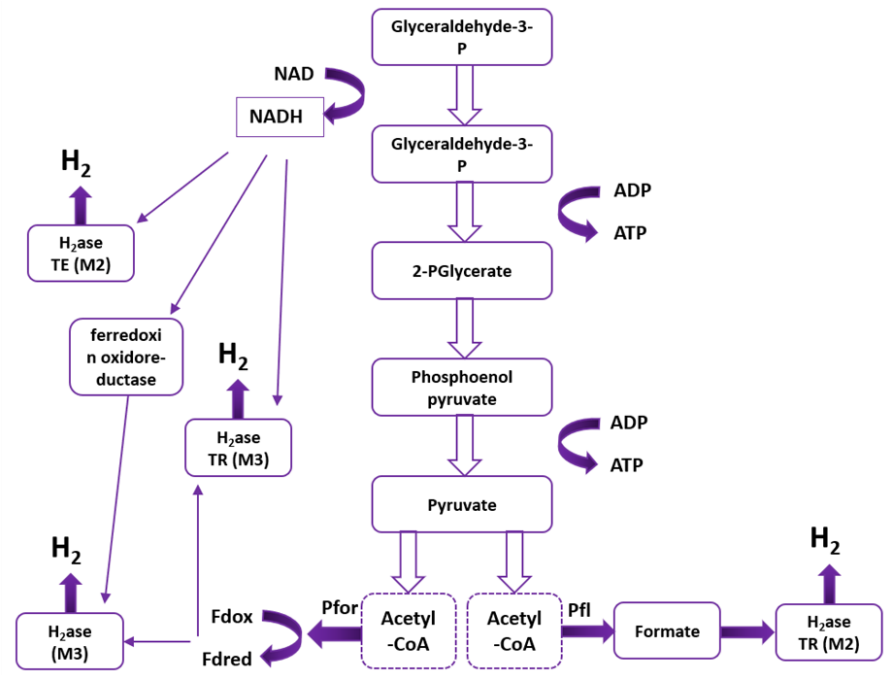


Fig. 2. Different types of hydrogenases in Clostridia to glycolysis and pyruvate degradation. Modified from Constant and Hallenbeck, (2019).

In this way, various investigations have been carried out to several experiments to find the optimal doses of TE to enhance the production of hydrogen (Table 3). Low and high concentrations of these TE may cause low hydrogen yields and production rates because of nutrient limitations and the inhibitory effects of high concentrations; for this reason, optimization of essential nutrient concentrations for different substrates and microbial cultures is an important issue; this intention can be assessed by varying its concentration within a desired interval where all other factors are kept constant (Argun and Onaran, 2017).

Table 3. Effect of trace elements on bio-hydrogen production.

Substrate	Inoculum	Trace element used	Concentrations	Reactor type and operational conditions	Yield	Reference
Acid hydrolysed WPT hydrolysate was used as substrate	Anaerobic sludge heat-treated	Fe added as $FeSO_4*7H_2O$	C/N/P/Fe ratio 100/5/9/0.278	Batch pH: 6.7 Temperature: 37 °C	0.656 mol H_2/mol glucose	(Argun and Onaran, 2017)
Cheese whey wastewater	Granular anaerobic heat-treated	Co, Ni, Zn, (1.3 mg/L and Fe (50 mg/L)		Batch pH= 5.5 Temperature: 36 °C	3.5 mol H_2/mol lactose consumed and 218.6 ml H_2/g lactose consumed	(Azbar *et al.*, 2009a)
Waste activated sludge	Anaerobic digested sludge heat-treated	Ni^{2+}	5 mg/L	Batch pH: 7.0 Temperature: 35 °C.	0.48 mL H_2/mg COD	(Chen *et al.*, 2021)
The experiments were performed	Anaerobic sludge heat-treated	Fe added as Fe_2O_3	0.1 g Fe_2O_3/dm^3	Batch pH: 5.5 Temperature: 35°C.	200 dm^3 H_2/kg VS	(Cieciura-Włoch *et al.*, 2020)

using sugar beet pulp						
Sugar beet pulp	Anaerobic sludge heat-treated	Fe added as Fe_2O_3	1 g Fe_2O_3/dm^3	Semi-continuous Temperature 35 ± 1 °C.	52.11 dm^3 H_2/kg VS	(Cieciura-Włoch *et al.*, 2020)
Fruit and Vegetable Wastes		Fe, Co, Ni, Zn	Fe (7.5), Co (8.71), Ni (29.48), Zn (79.76) mg/L	Batch Initial pH:7 Temperature: 55 ± 1 °C.	31-76 mL H_2/g VS	(Keskin *et al*., 2018)
OFMSW	*E. coli* cell suspension and *Enterobacter aerogenes* cell suspension	Fe as ferric oxid	100 mg/L	Batch pH: 5.5 Temperature: 37°C	872.5 ± 10.1 mL and 58.7 mL H_2/g Carbohydrates $_{initial}$	(Sharma and Melkania, 2018d)
Glucose	Sewage sludge heat-treated	Fe	200 mg Fe^{2+}/L	Batch pH: 5.5 Temperature: 37 °C.	217.4 ± 4.2 ml H_2/g glucose	(Zhang *et al*., 2017)

According to the literature review, different concentrations of TE have been proposed to increase hydrogen production. As reported by Soltan *et al*., (2019) the optimum dosages of Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+} and Na^{+} required for H_2 production were previously recorded in the range of 3000, 100–300, 100–600, 12 and 350–1000 mg/L, respectively. Meanwhile, Chen *et al.,* (2021) indicates that adding a concentration of 5 mg Ni^{2+}/L to dark fermentation process using waste active sludge as substrate can increase cumulative H_2 production by 29%, however, 0.1 mg/L Ni^{2+} concentration was found optimum for H_2 generation from glucose (Wang and Wan, 2008). There is no optimal dose of TE that can be applied in a general way since it is strongly dependent on the types of microorganisms present in the inoculum and the substrate used. Therefore, experiments carried out with different TE, on different substrates, and under different conditions are important to identify the doses that can increase hydrogen yields (Keskin *et al*., 2018), mainly in complex substrates such as OFMSW.

Different doses of nickel have been tested to stimulate the DF process from substrate easily degradable such as glucose and sucrose; however, it is necessary to understand the effect of nickel on more complex substrates as OFMSW. Fig. 3 shows the information reported in the literature for the hydrogen yields obtained by using nickel to stimulate the DF process (Bing-Feng *et al.*, 2009; Chen *et al*., 2021b; Li and Fang, 2007a; Lin and Lay, 2005a; Mullai *et al.*, 2013a; Taherdanak *et al*., 2016a; Wang and Wan, 2008b) The Ni^{2+} concentrations were adjusted for each gram of volatile solids of inoculum ($VS_{inoculum}$) added to the reactors since the Ni^{2+}/VS ratio led to the possible stimulation/inhibition effect on the process (Hickey *et al.*, 1989).

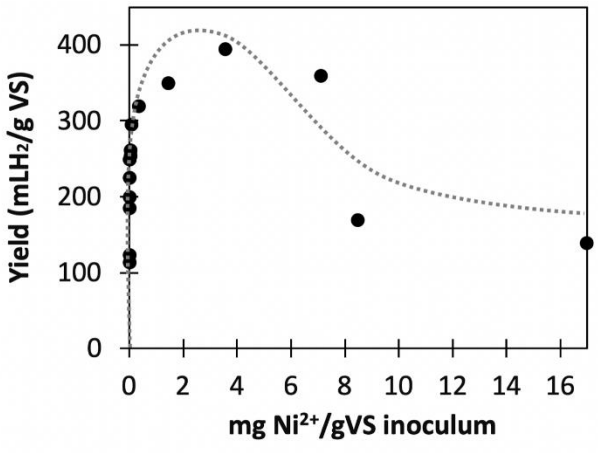


Fig. 3. Hydrogen yield and Haldane adjustment of added nickel concentrations per gram of inoculum (as volatile solid) from different authors (from Salazar-Batres and Moreno-Andrade, (2022)).

The highest hydrogen yields were obtained between 0.5-4 $mgNi^{2+}/gVS_{inoculum}$. When nickel concentrations increase, performance decreases due to the inhibition of microbial communities exposed to high concentrations of this metal; or it also may be due to nickel causing a radical change in fermentation towards amino acids pathway and away from carbohydrate metabolism. Concentrations above 1 mg/L of nickel generated a hostile environment for the methanogenic process. In comparison, some hydrolytic bacteria species are inhibited when nickel concentrations higher than 12 mg/L are present in the digesters (Ashley *et al.,* 1982).

2.3 Methane production (Anaerobic Digestion)

Anaerobic digestion (AD) is a biological conversion process without an external electron acceptor such as oxygen (Angelidaki and Sanders, 2004; Metcalf & Eddy, 2003). It involves sequential and parallel steps known as hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Choong *et al.,* 2016; Li *et al.,* 2019). These steps are carried out by groups of microorganisms e.g. fermenting bacteria, syntrophic acetogens, homoacetogens, and methanogenic archaea (Li et al., 2012). AD is an attractive technology to produce methane-rich biogas and suitability for the integration of a wide variety of process configurations and scales (Fermoso *et al.,* 2015). Various wastes have been used as substrates to generate methane-rich biogas, which may be transformed into electrical or thermal energy; thus, an enhancement of efficiency in methane generation from different substrates is proportional to more energy recovery in the anaerobic digestion (Li *et al*., 2019; Nevzorova and Kutcherov, 2019).

2.3.1 Use of trace metals in anaerobic digestion

Many kinds of substrates have been used in AD process for biogas production, including waste-activated sludge, food waste, farm waste, agricultural waste, and wastewater (Li *et al.,* 2019). However, some waste such as energy crops, agroindustry residues, and food waste are poor in TM (Demirel and Scherer, 2011; Schmidt *et al.,* 2014). In addition to the above, TM suitability strongly depends on the type of substrate (Abdelsalam *et al.,* 2017a). TM addition is needed to achieve higher methane yields, especially in mono-digestion. Many studies have been performed using different types of waste mostly food waste (Banks *et al*., 2011; Capson-Tojo *et al.,* 2018; Facchin *et al.,* 2013; Ko *et al.,* 2018; Parra-Orobio *et al.,* 2018; Qiang *et al.,* 2013, 2012; Zhang and Jahng, 2012; Wanli Zhang *et al.,* 2015; Wanqin Zhang *et al.,* 2015), sewage sludge, waste-activated sludge (Ashley *et al*., 1982; Hickey *et al.,* 1989; Linville *et al.,* 2016; Zhang *et al.,* 2014; Zhen *et al.,* 2015), maize silage (Evranos and Demirel, 2015; Gustavsson *et al.,* 2011, 2011; Pobeheim *et al*., 2010; Wall *et al.,* 2014), slurry (Abdelsalam *et al.,* 2017b), methanol (Zandvoort *et al*., 2003), wheat stillage (Schmidt *et al.,* 2014) and molasses stillage (Espinosa et al., 1995). Micronutrients such as TM are associated with reactor performance. TM insufficiency may result in a negative effect on

potential biomethane yields (Wall *et al.*, 2014). Hence, effective biogas production requires optimal conditions of temperature, partial pressure, pH, hydraulic retention time, nature of substrate, adequate C/N ratio, stirring intensity, trace elements concentration, microbes balance, and digester size (Fermoso *et al.*, 2015; Garuti *et al.*, 2018; Sambo *et al.*, 1995). TM supplementation responses also depend on several factors, including the composition of the substrate, the source of inoculum, or the operational mode of an anaerobic digester (Hinken *et al.*, 2008). Table 4 compiles the TM concentrations added to the AD process in addition to the operating conditions such as pH, temperature, stirring, type of reactor, and operation.

As a result of the complex aqueous chemistry and syntrophic biological processes, TM bioavailability is affected by operational parameters such as pH since it can affect the concentration of metals in the solution phase (Thanh *et al.*, 2016; Filgueiras *et al.*, 2002). Metals precipitation or chelation may occur due to pH or buffering capacity adjustment through the external addition of certain chemicals (Evranos and Demirel, 2015). In the research carried out by Callander and Barford (1983), they concluded that metal solubility increases when the pH levels change from 7.0 to 7.5. The experiments carried out by Lo *et al.*, (2009) proved that TM such as Cr, Ni, and Zn have different solubility levels according to pH. Different pH values have been reported in research related to the use of TM to improve AD process performance in batch tests (Banks *et al.*, 2011; Capson-Tojo *et al.*, 2018). According to Fermoso *et al.*, (2015), an increase in the pH value can decrease cationic metals. The reactor stirring plays a significant role in methane production. Different stirring regimes have been reported including continuous stirring (Nordell *et al.*, 2016; Qiang *et al.*, 2013; Wanli Zhang *et al.*, 2015), manual agitation (Ko *et al.*, 2018; Parra-Orobio *et al.*, 2018), and intermittent agitation (Ashley *et al.*, 1982; Capson-Tojo *et al.*, 2018; Gustavsson *et al.*, 2011; Noonari *et al.*, 2019; Pobeheim *et al.*, 2010). Stirring influences the distribution of nutrients, substrate, and microorganisms leading to the TM distribution in the reactor and preventing precipitation (Heyer *et al.*, 2020; Liu *et al.*, 2018; Wang *et al.*, 2017).

The effect of TM may be different according to the inoculum characteristics and origin since the microbial population could be different (De Vrieze *et al.*, 2015). It is crucial to consider the initial inoculum characteristics such as TM content, nutrients, and enzymatic activity of inoculums when the effects of different inoculums in AD are compared. The substrate degradation and biogas production is frequently studied; nevertheless, the composition and characterization of the inoculums are not investigated (Gu *et al.*, 2014). Parra-Orobio *et al.*, (2018) studied the effect of inoculum (from a municipal and two agro-industrial wastewater treatment plants) in AD of food waste and concluded that the TM concentration in the inoculum had an essential impact on the performance of the AD processes operated in batch mode or at the early stages of a semicontinuous digester operation.

Table 4. Summary of the operating conditions in the treatment of organic waste using trace metals

Regimen	pH	Temp. (°C)	Stirring	HRT (d)	OLR	Substrate	Inoculum	TM addition	Ref.
Batch	7	37	50 rpm (6 min, 3 times/h)			Primary settled sludge		25, 50, 100, 150 and 250 mg Ni/L	(Ashley *et al.*, 1982)
	7	37				Synthetic mixture of butyric 28 mmol/L VFA, propionic 116 mmol/L VFA and acetic 213 mmol/L VFA.		Ni (1.86–199), Co (3.07–3.58), Se (0–0.11) and Mo (0.76–1.17) (mg/L)	(Ezebuiro *et al.*, 2018)
	8	36	60 rpm			A mix of propionic, acetic acid, glucose, starch, and ammonia	lab. food waste digester	Se 0.2, Mo 0.2, Co 1, and W 0.2, (mg/L)	(Banks *et al.*, 2011)
	7	37	70 rpm, 1 min every 30 min			Canola straw with buffalo dung, and banana waste plant with buffalo dung	continuous stirred tank reactor (CSTR)	0.4, 0.5, 0.81, 1.22, and 1.63 mg of Fe_3O_4 magnetite nanoparticles	(Noonari *et al.*, 2019)

	8.1	37	40 rpm (1 min, every 10 min)			Food waste	industrial plant treating different organic streams	Fe 100, Co 1, Mo 5, Ni 5, Se 0.2, Cu 0.1, Mn (mg/L)	(Capson-Tojo *et al*., 2018)
	7-8	37	400 rpm (15 min, 4 times/d)			Wheat stillage	pilot scale biogas plant	Co 0.5, Ni 0.2, and Fe 500 (mg/L)	(Gustavsson *et al*., 2011)
	8	35	Manual stirring twice a day			Food waste	secondary sedimentation tank	1 mg Ni^{2+}	(Ko *et al*., 2018)
	7	35	Intermittent manual stirring	32		Food waste	Sugar industry Slaughter of cattle and pigs Municipal wastewater treatment plant	Ca, K, Fe, Zn, Al, Mg, Co, Ni, and Mo	(Parra-Orobio *et al*., 2018)
	7-8	37	150	20		Food waste		Fe 100, Co 1, Mo 5, and Ni 5 (mg/L)	(Wanli Zhang *et al.*, 2015)
	7	35	15 min, 8 times/d	30		Maize silage	an agricultural biogas plant	10.6 µM of Ni and 2.0 µM of Co	(Pobeheim *et al*., 2010)
CSTR		39	100-200 rpm		2.4, 3.3 Kg VS/m^3/d	Manure and industrial waste	biogas plant	Fe, Co, Ni, Se and W	(Nordell *et al.*, 2016)
	6.9-7.5	55	1400 rpm	30	6.3 kg COD/ m^3/d	Food waste	thermophilic anaerobic digester sludge from a municipal sewage treatment plant	276 mg Fe/kg COD removed, 4.96 mg Co/kg COD, 4.43 mg Ni/ kg COD	(Qiang *et al.*, 2013)
		37			1.7 6.3 kg COD/ m^3/d	Glucose, sucrose, lysed casein, VFA, and alcohols	Digestate from a reactor treating slaughterhouse waste	0.36 ng Co/g COD fed, 0.15 ng Ni/g COD fed, 0.11 ng Se/g COD fed, 0.28 ng W/g COD fed	(Šafarič *et al.*, 2020)
		37-55				H_2/CO	Anaerobic sludge from a landfill	4 g $(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$, 1 g $CoCl_2 \cdot 6H_2O$ and 0.1 g $NiCl_2 \cdot 6H_2O$	(Li *et al.*, 2020)
UASB	7	30	70 rpm	0.5	2.6-7.8 g COD/ m^3/d	Methanol	Granular sludge from a full scale UASB reactor	Trace element solution: Fe 562, Ni 32, Zn 24, Mn 139, Cu 14, Co 495, Mo 27, Se 49 (mg/L)	(Zandvoort *et al*., 2003)

*Working volume, ** Headspace

2.3.2 Optimal doses of trace metals for methane production

The TM are components of cofactors in enzyme systems, the most essential in AD are Fe, Ni, and Co (Thanh *et al.*, 2016; Ünal *et al.*, 2012; Zhang and Jahng, 2012). Methanogenic archaea require large amounts of these TM and a smaller amount of Mo or W and Zn (Glass and Orphan, 2012). Some Ni or Co ion-containing enzymes involved in methanogenesis have been identified (Kida *et al.*, 2001). Ni^{2+} is the center of coenzyme F_{430} in methyl coenzyme M reductase (MCR) that catalyzes the methyl-CoM to CH_4 in methanogenesis (Grabarse *et al.*, 2001; Moore *et al.*, 2017; Zhang *et al.*, 2009). Co is present in cyanocobalamin required for the synthesis of vitamin B12, and it participates in several reactions in both the hydrogenotrophic and acetoclastic methane formation (Ko *et al.*, 2018; Myszograj *et al.*, 2018; Schattauer *et al.*, 2011).

Otherwise, enzymes [Ni-Fe]- hydrogenases are involved in methanogenesis, and each one contains abundant Fe (Glass and Orphan, 2012; Shima *et al.*, 2011; Thauer *et al.*, 2010). Fe is also an electron acceptor in cytochromes, participating in the synthesis of catalase, peroxidase, and aconitase, it also has redox properties (Schattauer *et al.*, 2011). Ignace *et al.*, (2016) reported that Fe could help to recover methane better and more efficiently from sludge. Fe has also been added to anaerobic reactors as zero-

valent iron nanoparticles since it influences the generation of H_2 through Fe^0 which produces methane, thereby increasing methane production (Li *et al.,* 2012). Molybdenum is present in the enzyme formate dehydrogenase (FDH) which catalyzes formate production (Myszograj *et al.,* 2018), only when Mo is present in the growth medium the Mo enzyme is synthesized (Thanh *et al.,* 2016). TM are essential for biochemical reactions, enzyme activities, methane yield, and volatile fatty acids (VFAs) utilization (Ko *et al.,* 2018).

The effects of metals highly depended on the supplemental concentrations since appropriate concentrations of TM are required for optimal process performance (Wanli Zhang *et al.,* 2015). The supplementation of necessary TM is useful to improve AD performance (Choong *et al.,* 2016). Several concentrations of TM have been proposed; however, the results obtained are not homogenous since when TM are supplemented at high concentrations, metals may act as inhibitors affecting the enzymes' functions (J.A.Oleszkiewicz, 1990). Feng *et al.,* (2010) evaluated the effect of three TM and their combined effects on a tank reactor biogas process with an OLR of 4.0 gVS/L/d using food waste as substrate. The highest predicted methane generation (860 mL CH_4/gVS) occurred by applying Se: 0.8 mg/L, W: 1.8 mg/L, and Co: 0.06 mg/L. Gustavsson et al., (2011) investigated the effect of TM addition on lab-scale biogas tank reactors using wheat stillage as substrate, to maintain the process stability daily supplementation with Co (0.5 mg/L), Ni (0.2 mg/L), and Fe (0.5 mg/L) was needed; the operational conditions were an HRT of 20 d and an OLR of 4 gVS/L/d. They concluded that to maintain a stable process it was necessary to add cobalt and nickel.

Trace metals concentrations applied to reactors operating under different conditions vary significantly. Espinosa *et al.,* (1995) used large amounts of TM (Fe 100 mg/L, Ni 15 mg/L, Co 10 mg/L, and Mo 0.2 mg/L) in a UASB reactor fed with vinasse at high organic loads over 17 kg COD/m^3/d, while in case of Gustavsson *et al.,* (2011), a lower amount of trace metals were used (Co 0.5 mg/L, Ni 0.2 mg/L, and Fe 0.5 g/L) to maintain the process stability at the organic loading rate of 4.0 g VS/L/d. For this reason, it is necessary to determine the minimum dose of TM with which the systems remain stable since the use of salts such as $NiCl_2$ and $FeCl_3$ could have adverse environmental effects, according to the case study carried out by Hijazi *et al.,* (2020), who carried out a life cycle analysis of the salts added to anaerobic reactors.

2.4 Biogas production in a two stages system from OFMSW

OSAD has limitations since all the steps (hydrolysis to methanogenesis) occur in a single environment. Two major groups of microorganisms are kept together, and the differences in their nutritional needs, physiology, and growth kinetics could reduce the overall AD performance and bioenergy recovery (Azizi *et al.,* 2019). Furthermore, OSAD should lead with challenges such as volatile fatty acids (VFA) accumulation, insufficient buffering capacity, production of harmful intermediates which reduce the system stability (Srisowmeya *et al.,* 2020). Due to the potential of the AD process to recover energy from OFMSW, there are much research focused on improving process conditions and generating better yields (Chowdhury *et al.,* 2020; Corsino *et al.,* 2021; Ghanimeh *et al.,* 2020; Mu et al., 2018). In the case of De Vrieze *et al.,* (2013), who operated a semi-continuous reactor under mesophilic conditions, they obtained a productivity of 1.15±0.22 L CH_4/L/d; however, the process failed, and the use of a co-substrate was needed. Ghanimeh *et al.,* (2012), operated a batch reactor under thermophilic conditions using OFMSW as substrate; a specific methane yield (SMY) of between 314 and 327 mL CH_4/gVS were obtained. Nevertheless, it was detected an accumulation of acetic acid and propionic acid, which was controlled applying slow agitation to the reactor. In the case of Chowdhury *et al.,* (2020), obtained a SMY of 210 mL CH_4/gVS by operating a reactor with a high solids load, requiring a second stage by ultrasonication of digestate and wet-type anaerobic digestion for effective biomethane recovery, after the post treatment, it was possible to produce up to 132 mL CH_4/gVS.

Maintaining the stability of the OSAD from OFMSW process is challenging and requires the use of certain strategies to enhance the biogas production. For this reason, two-stage AD process (TSAD) and trace

metals addition (TM) have been tested to provide optimal growth conditions for microorganisms. In TSAD, hydrolysis and acidogenesis are carried out in a different reactor (De Gioannis *et al.,* 2017; Voelklein *et al.,* 2017). During hydrolysis, complex organic polymers are hydrolyzed into simple soluble organic compounds; subsequently, the generation of volatile fatty acids, H_2-rich biogas (H_2 and CO_2), and other intermediates occurs during the acidogenesis (Angeriz-Campoy *et al.,* 2018). In the TSAD carried out by Xiao *et al.,* (2019), from food waste (FW) and a co-digestion of FW and paper waste, it was obtained SMY of 460 and 360 mL CH_4/gVS, respectively. Similarly, Voelklein *et al.,* (2017), compared the SMY in one and two-stages; in the case of the OSAD, a yield of 82.7 mL CH_4/gVS was obtained, observing the significant deterioration of the parameters pH, volatile fatty acids/total alkalinity ratio and VFA. This decline in the system was reduced after TM addition. As a result of the TSAD, yields of up to 419±23 mL CH_4/gVS were obtained. The enhancement in the SMY was attributed to a higher hydrolysis rate in the first stage.

3 THESIS STATEMENT-JUSTIFICATION

Anaerobic digestion process has been widely studied, even though there are still improvement opportunities for upgrading the performance of this biological process. Such is the case of trace metals addition since its application can stimulate the anaerobic digestion and dark fermentation processes. TM acts as cofactors in numerous enzymes involved in these processes. For this reason, it is necessary to determine the doses of TM to enhance biogas production, avoiding accumulation and toxic effects. Research related to TM has been focused on the stages of acetogenesis and methanogenesis. Therefore, it is important to explore the TM influence in hydrolysis and acidogenesis since the system requirements at each stage may differ.

4 HYPOTHESIS

Trace metals supplementation in hydrogen-producing reactors will increase the production of hydrogen-rich biogas, while acidogenic effluents enriched with trace metals will improve the stability of methane-producing reactors.

5 GENERAL AND SPECIFIC OBJECTIVES

5.1 General Objective

To propose the optimal Ni^{2+} and Fe^{2+} concentrations that increase the productivity and yield of hydrogen and methane in sequencing batch reactors using organic solid waste as substrate.

5.2 Specific Objectives

1. To evaluate the effect of Ni^{2+} addition on producing H_2-rich biogas and volatile fatty acids in batch systems and sequencing batch reactors from organic solid waste.

2. To evaluate the increase in biogas (H_2 and CH_4) and possible changes in microbial communities derived from Ni^{2+} addition in a two-stage sequencing batch system compared with a conventional anaerobic digester.

3. To evaluate the effect of Fe^{2+} and Ni^{2+} addition on H_2 and CH_4 production in a two-stage process using organic solid waste as substrate.

6 METHODOLOGY

The experiments were divided into two main sections (Fig. 4). In the first section, the Ni^{2+} effect was studied in the DF process using glucose and OFMSW as substrate, and in a two-stage process comparing the results with a conventional process (AD single stage). In the first stage, different concentrations of Ni^{2+} were added, and the effluents were used to feed the methanogenic reactor. The results were compared with the methane yields and productivities obtained in a conventional AD process. Section 2 corresponds to the study of the Ni^{2+} and Fe^{2+} effect (Fig. 5). To achieve this objective, i) different concentrations of TM were tested in batch process, and ii) the TM concentrations that enhanced the biogas production were applied in a two-stage system. The details of the TM concentrations used in the experiments are reported in the following sections.

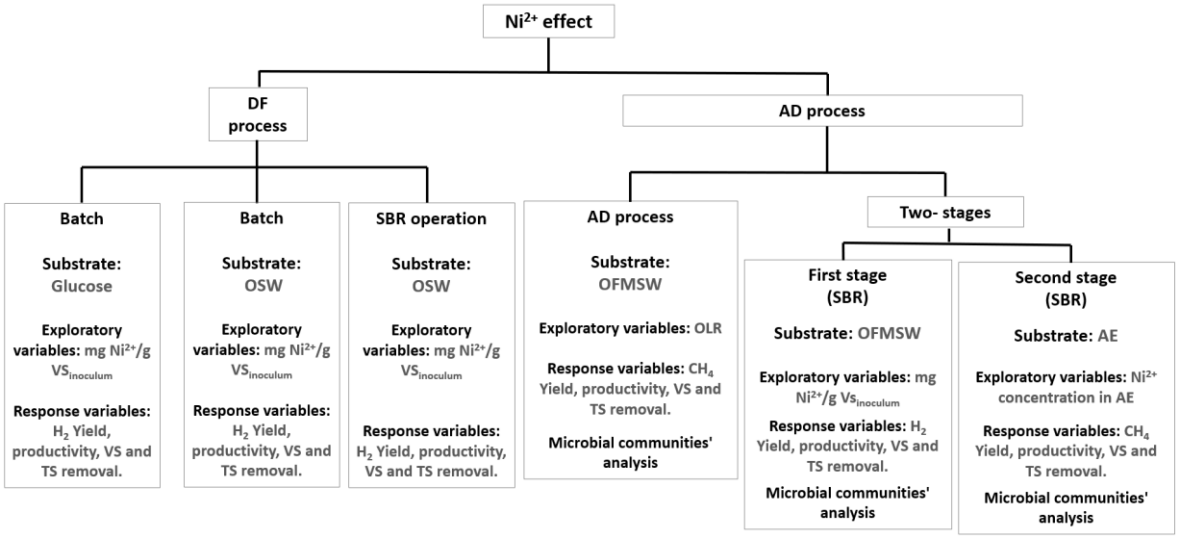


Fig. 4. Diagram of methodology, section 1.

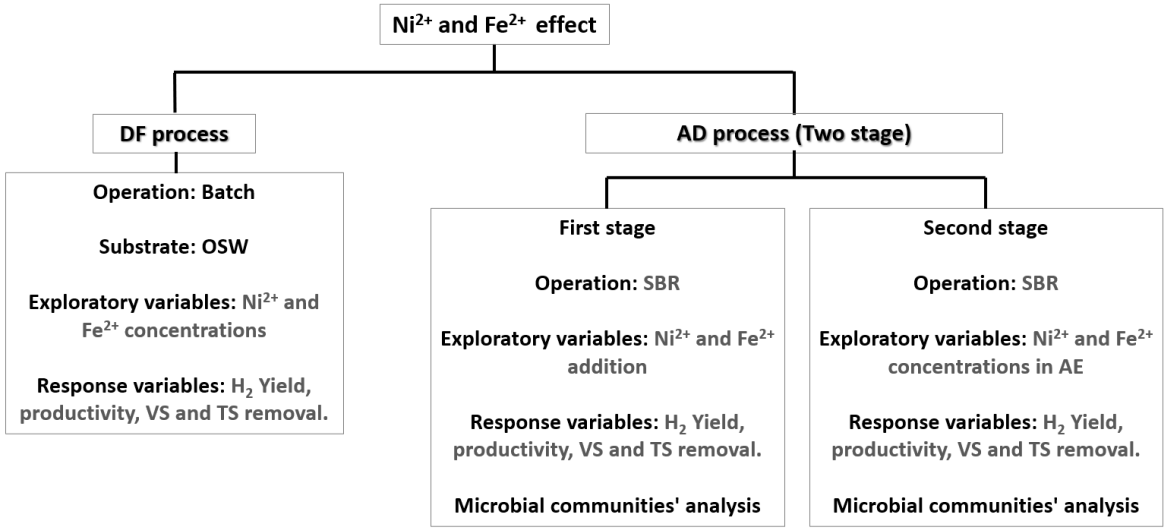


Fig. 5. Diagram of methodology, section 2.

6.1 Characterization of the substrate and inoculum

The OFMSW was obtained from the regional municipal market of Queretaro, Mexico, it was shredded in an industrial blender, stored in plastic bags, and finally frozen at -4 °C until use. The OFMSW was composed of papaya, green vegetables, cucumber, radish, tomato, grape, mango, and banana, with TS and VS of 10 and 8%, respectively, and a nickel concentration of 0.17 ± 0.06 mg Ni/g TS. Another sample of OFMSW was collected from an urban solid waste separation plant located in Queretaro, Mexico (solid waste was delivered to the plant as a source segregated at the household level in an area with a population of 1 million inhabitants). The OFMSW contained 78.8% of organic solid waste and the rest nonfermentable material: paper 12.2%, plastic 5.1%, glass 3.0%, metal 0.2%, wood 0.5%, and bones 0.2%. Consequently, a manual separation was carried out to obtain a sample without inert materials. The sample without inert materials was ground in an industrial mill to obtain a particle size of <0.2 mm. The OFMSW presented the following physicochemical parameters: density (g/mL) 1.05, pH 5.93, TS and, VS (g/Kg), 507, 250, respectively, and total COD (g/L), 483.42. Fe and Ni concentration were 8.7±0.05 mg Fe/gTS and 0.2±0.04 mg Ni/gTS.

The inoculum corresponds to anaerobic granular sludge recovered from an up-flow anaerobic sludge blanket digester; it was stored at 4°C until use. For acidogenic reactor's inoculation, the granular sludge was thermally pretreated at 105°C for 24 hours to eliminate the hydrogen-consuming methanogens (Buitrón and Carvajal, 2010) and select the microorganisms that form spores such as bacteria from the genus *Clostridium* which are considered the most efficient H_2-producers (Castelló *et al*., 2020). For the anaerobic digester operation, the methane biochemical potential assay, and the methanogenic reactor (second stages) the anaerobic granular sludge was used without pretreatment. The anaerobic granular sludge presented a composition of 183±28 gTS/Kg and, 171±25 gVS/Kg, while the pretreated granular sludge had a composition of 980 gTS/Kg and 780 gVS/Kg. Fe and Ni concentration were 0.82±0.02 mg Fe/gTS and 0.0018±0.04 mg Ni/gTS.

6.2 Analytical methods

Chemical oxygen demand (COD) was determined by HACH reactor digestion method. Total and volatile solids (TS and VS) were measured in triplicate using the standard methods 2540 G (APHA, 2017). Total carbohydrate was measured using a phenol-sulfuric assay (DuBois et al., 1956). The quantification of the volatile fatty acids (VFA) was carried out according to Cardeña *et al*., (2017) by gas chromatography with a flame ionization detector (FID, Agilent Technologies 7890B), equipped with the DBFFAP column of 15 m and 1 µm of the thickness. The mobile phase corresponds to nitrogen gas with a flow of 25 mL/min. The temperature of the injector and the detector corresponds to 190 and 210 °C, respectively. The biogas was measured with a compact standalone volumetric gas flow meter (BPC µFlow) to normalize gas flow rate and volume measurement at 0 ˚C and 1 atmosphere. Biogas composition was determined by gas chromatography using an equipped SRI 8610C chromatograph with a thermal conductivity detector and a 30 m Carboxen 1010 column (ID 0.53 mm). The injector, column, and detector temperatures were 200, 100, and 230°C, respectively. The mobile phase corresponds to nitrogen gas with a 4 mL/min flow rate. Nickel and iron in the substrate, inoculum, and digestates was quantified according to the standard methods (3111A) by flame absorption spectrometry.

6.3 Microbial community analysis and data analysis

Samples were taken to analyze planktonic microbial communities from the acidogenic and methanogenic effluents. All the samples were stored at -20 °C. Genomic DNA was extracted using the DNeasy Power Soil Kit (Qiagen, Germany). The DNA quality was verified by spectrophotometry (NanoDrop™ 2000c, Thermo Scientific, USA). Partial bacterial and archaeal 16S rRNA genes were PCR-amplified with the primers 515F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGYCAGCCGCCGCGGTAAHACCVGC-3′) and 806R (5′- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACVSGGGTATCT-

AAT-3′) with Illumina adapter (Caporaso *et al*., 2012). The PCR reaction was prepared using 19 µL sterile distilled water, 0.5 µL 0.2 µM primer, 0.25 µL 25 µM $MgSO_4$, 0.25 µL 20 mg/L bovine serum albumin, 2.5 µL 10X PCR buffer, 0.1 µL of Ex Taq polymerase high fidelity and, 2 µL of template DNA. The PCR reaction was performed as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles or 38 cycles (for acidogenic or methanogenic samples, respectively), 95 °C for 30 s, 55 °C for 30 s, 68 °C for 30 s, and final extension at 68 °C for 10 min. The PCR amplicons were verified on agarose gel electrophoresis. The PCR products were cleaned using the kit AMPure XP beads (Beckman Coulter Inc., Brea, CA, USA). A second PCR was done to integrate the barcode and Illumina adaptor (Illumina, 2013) required for sequencing libraries. The PCR products were cleaned using the kit AMPure XP beads (Beckman Coulter Inc., Brea, CA, USA) before their quantification was performed using the kit Quant-IT^{TM} PicoGreen® dsDNA Assay (Invitrogen-P7589). Finally, the pool of sequencing libraries was shipped to the Centre d'Expertise et de Services Génome Québec (Montréal, Québec, Canada) for sequencing with the Illumina MiSeq PE-250 platform.

PCR primer sequences were removed from raw reads using the tool Cutadapt (v.2.1) (Martin, 2011). Sequences were processed in R (v. 4.2.1) using the DADA2 package (v. 1.20.0) (Callahan *et al*., 2016). Default arguments were kept for sequence processing, except for the trimming of forward and reverse sequences at 230 bp and 230 bp based on the sequence quality. The taxonomic affiliation of bacterial and archaeal ASV was assigned with the Silva database (v. 138.1) (Quast et al., 2013). Diversity analysis was performed in R with the phyloseq package (v. 1.36. 0) and ggplot package was used to produce the graphs (McMurdie and Holmes, 2013; Villanueva and Chen, 2019). In order to explore the dissimilarities among the microbial communities and the samples, it was performed the analysis of distance-based redundancy analysis (dbRDA) (Legendre and Anderson, 1999). Also, an analysis of compositions of microbiomes with bias correction (ANCOM-BC-2) was performed to estimate the unknown sampling fractions and corrects the bias induced by their differences among samples (Lin and Peddada, 2020) and the results were used to create a volcano plot to visualize the significant ASV differences among the samples (Mullan *et al*., 2021). Raw reads were deposited to Sequence Read Archive repository of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/bioproject/910650, https://www.ncbi.nlm.nih.gov/sra/PRJNA923699) in the BioProjects PRJNA910650 and PRJNA923699.

6.4 Effect of nickel concentration on biohydrogen production: organic solid waste vs. glucose

To develop the specific objective 1, the first set of experiments was carried out to test the effect of nickel on biohydrogen production from OFMSW. In particular, the experimental setup was focused on determining the dose of nickel that must be added to each gram of volatile solid of inoculum to stimulate the hydrogen yield (HY) and productivity in an H_2-producing reactor using OFMSW as substrate.

6.4.1 Batch experiments to test the effect of different concentrations of Ni^{2+}

To examine the biohydrogen potential (BHP), automatic methane potential test (AMPTS II) equipment was used, which measures the final specific production for a maximum degradation time; the BHP tests were performed according to the protocol established by Carrillo-Reyes *et al*., (2019). The temperature of the batch tests was 37±1 °C, and the initial pH was adjusted to 7.5±0.2. An experimental arrangement was carried out to test six different Ni^{2+} concentrations added as $NiCl_2$•$6H_2O$ (0, 0.1, 0.5, 1, 1.5, 2, and 5 mg Ni^{2+}/g $VS_{inoculum}$) using OFMSW and glucose as the carbon source. The mg Ni^{2+}/g $VS_{inoculum}$ concentrations mentioned above were determined based on the literature review (Fig. 3). The H_2 cumulative volume was adjusted to the modified Gompertz equation shown in Equation (1), with H_{max} (in mL) representing the H_2 volume, R_{max} (in mL/min) representing the maximum flowrate, a lag period λ (in min), and the steady flowrate after peaking mt (in mL/min) (Jiménez-Ocampo *et al*., 2021; Santiago *et al*., 2020); the results were analyzed by using MATLAB R2021b. A three-way ANOVA was carried out in

Rstudio software version 1.4.1106 considering the substrate and Ni^{2+} concentrations and their interactions concerning HY.

$$H(t) = H_{max}\exp\left[-exp\left(\frac{2.71828*R_{max}(\lambda-t)}{H_{max}}+1\right)\right] \quad \text{(Eq. 1)}$$

6.4.2 Effect of Ni^{2+} on H_2 production in a Sequencing Batch Reactor

The hydrolytic-acidic reactor consisted of an acrylic cylinder with a volume of 1.5 L operated under mesophilic conditions (37±1 °C) by recirculating water with a heating circulator with open bath equipment (Thermo Scientific). Masterflex pumps were used for filling and discharging the substrate and digestate. The reactor was operated as a Sequencing Batch Reactor (SBR) with the following times for the operation phases: filling, 4.3 min; reaction, 23 hours; sedimentation, 51 min; and discharging, 4.7 min. The organic loading rate (OLR) was 20 g VS/L/d, and the hydraulic retention time was 2 d. Fig. 6 shows the bioreactor setup. The substrate and inoculum used were OFMSW described in section 5.1 of the methodology. The reactor was inoculated with 1.85 g VS of pretreated inoculum, maintaining a substrate to inoculum ratio (S/I) of 2.7. The long-term effect of nickel addition on the bioreactor was divided into the following 3 phases: i) no nickel addition, ii) evaluation of the addition of 0.1 mg Ni^{2+}/g VS, and iii) evaluation of the addition of 0.5 mg Ni^{2+}/g VS. The reactor was operated for 30 cycles. The reactor stability was reached at cycle 15. Each condition was tested during five operational cycles. Effluent and biogas from each cycle were evaluated.

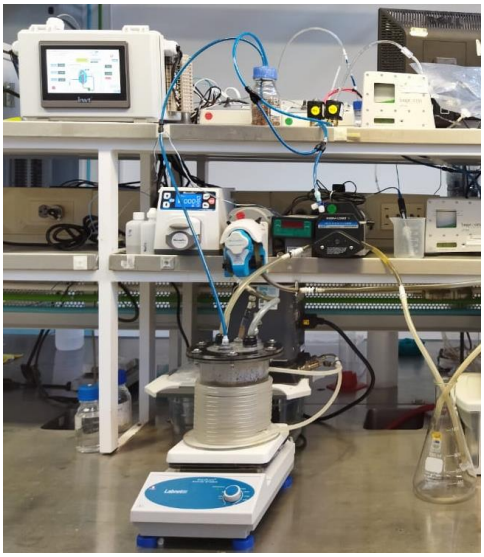


Fig. 6. Hydrogen producing reactor.

6.5 Biogas production from OFMSW is enhanced by nickel addition in a two stages system compared with a conventional digester

This section corresponds to the methodology to develop the specific objective 2. The biogas produced on OSAD was compared to the production on TSAD. In the first stage, the reactor was fed with OFMSW using an OLR of 60 gVS/L•d. Ni^{2+} was added in this stage in the following order: 0, 0.1, and 0.5 $mg/gVS_{inoculum}$. Three types of effluents were collected (namely acidogenic effluents 1, 2, and 3). Subsequently, the methanogenic reactor was fed with the 3 effluents. We also explored the impact of TSAD and OSAD and the addition of TM on microbial communities.

6.5.1 Conventional anaerobic digestor (AD conventional single stage)

A conventional anaerobic digester was operated in a sequencing batch reactor (SBR) in an INFORS HT model Labfors 5 reactor with a capacity of 3.2 L and a working volume of 2.8 L (Fig. 7). The settling, discharging, and feeding time were 50, 5, and 7 minutes, respectively. The feeding and discharge were fed with peristaltic pumps Masterflex Model 77200-60 and 77200-50, respectively. The reactor had a sensor for pH measurement, a paddle stirrer, and a heat exchanger. The operational conditions were a stirring speed of 80 rpm, pH of 7.5±0.2, and temperature of 37±0.5 °C. The HRT was 13 d and an OLR of 2.5 gVS/L·d, starting with an OLR of 1.5 gVS/L·d for the first four cycles. The reactor inoculation was carried out with a S/I of 2 in terms of VS. The reactor was operated for ten cycles until the biogas production was constant; after that period, the evaluation of the biogas production began for 14 cycles.

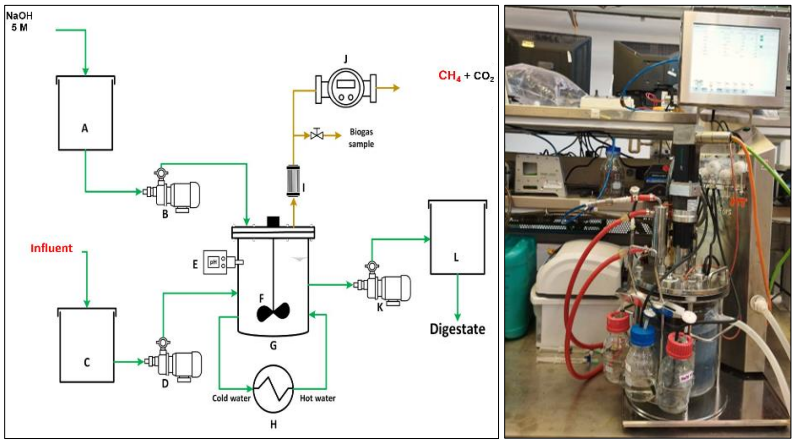


Fig. 7. Bioreactor setup: A) storage tank of the 1 N NaOH solution, B) 1 N NaOH addition pump, C) influent storage tank, D) influent feed pump, E) pH meter sensor, F) stirrer, G) SBR, H) Heated water recirculatory, I) Condensates trap, J) Flow meter, K) digestate discharge pump, L) digestate storage tank.

6.5.2 Two stages system for Ni^{2+} evaluation

6.5.2.1 Acidogenic digester (first stage)

The acidogenic sequencing batch reactor (SBR) used as first stage had a working volume of 1 L and a headspace volume of 0.5 L. The emptying and filling time were 5 minutes, while the settling and sedimentation time were 50 minutes. The feeding and discharge were performed with peristaltic pumps Model 77800-50 and 77200-62, respectively. The pumps were turned on and off with a programmable logic controller. The organic loading rate (OLR) was 60 gVS/L·d, and the hydraulic retention time (HRT) was 16 h. The temperature was maintained under mesophilic conditions at 37±0.5 °C by recirculating water with a heating circulator (open bath equipment Thermo Scientific). The stirring speed was 120 rpm, and the pH was 5.5±0.5. The inoculum/substrate ratio (S/I) used to inoculate the acidogenic SBR was 2.7. The reactor was operated for ten cycles until the biogas production was constant. The Ni^{2+} concentrations were determined based on the previous experiment (section 5.4 of methodology). Ni^{2+} was added as $NiCl_2 \cdot 6H_2O$. The evaluation of cycles 1 to 6 was without Ni^{2+} supplementation. In cycles 7 to 14 the concentration of 0.1 mg $Ni^{2+}/gVS_{inoculum}$ was tested. At last, the concentration of 0.5 mg $Ni^{2+}/gVS_{inoculum}$ was tested from cycles 15 to 32. Three effluents were collected for subsequent biochemical methane potential assay, namely 0, 0.1, and 0.5 mg $Ni^{2+}/gVS_{inoculum}$.

6.5.2.2 Biochemical methane potential assay (BMP)

After the fermentation process, a BMP was performed to evaluate the volume of CH_4 produced from each acidogenic effluent. The batch tests were tested as technical triplicate, according to the methodology established by Angelidaki *et al.* (2009) in the equipment Automatic Methane Potential Test System II (BPC instruments) (Fig. 8). The substrates correspond to the three acidogenic effluents collected from the

different conditions (0, 0.1, and 0.5 mg $Ni^{2+}/gVS_{inoculum}$). The batch reactors were inoculated with a S/I of 2 gVS/gVS. The experimental conditions were a temperature of 37±0.5 °C, initial pH of 7.5, and intermittent stirring at a speed of 120 rpm (1 minute on, 2 minutes off). The reaction time was 18.75 days.

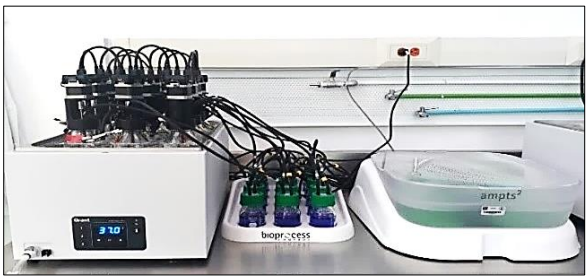


Fig. 8. Biochemical methane potential assay to test the acidogenic effluents.

6.5.2.3 Methanogenic digester (second stage)

The evaluation of the second stage to produce CH_4-rich biogas was carried out in the same reactor described in section 5.5.1 of the methodology, under the same operating conditions. In this stage, the three different acidogenic effluents previously homogenized were evaluated to test the effect of the acidogenic effluents enriched with Ni^{2+}. The reactor was operated for 15 cycles. The HRT was 6.52 d, and the OLR of the three operating conditions were 4.11, 3.04, and 2.32 gVS/L·d or 58.8, 44.0, and 38.2 gCOD/L·d. The reactor is presented in Fig. 9.

Fig. 9. Methanogenic reactor. (Second stage)

Fig. 10 is the representation of the two stages system for biogas production (hydrogen and methane). It was integrated by: 1. Storage tank, 2. Acidogenic Reactor Feed Pump, 3. Acidogenic reactor, 4. Magnetic stirrer, 5. pH sensor, 6. Magnetic stirrer, 7. Heated water recirculator, 8. NaOH storage tank, 9. NaOH addition pump, 10. Valve normally closed, 11. Biogas storage bag, 12. Valve normally open 13. Condensates trap, 14. Biogas sampling, 15. Check valve, 16. Biogas flow meter, 17. Discharge pump, 18. Acidogenic effluent storage tank, 19. Control system for acidogenic reactor, 20. Influent storage tank, 21. Methanogenic Reactor Feed Pump, 22. Methanogenic reactor, 23. Stirrer, 24. pH sensor, 25. Jacketed Glass, 26. NaOH storage tank, 27. NaOH addition pump, 28. Valve normally closed, 29. Biogas storage bag, 30. Valve normally open, 31. Condensate trap, 32. Biogas sampling, 33. Check valve, 34. Biogas flow meter, 35. Methane Reactor Discharge Pump, 36. Storage tank, 37. Control system for methanogenic reactor.

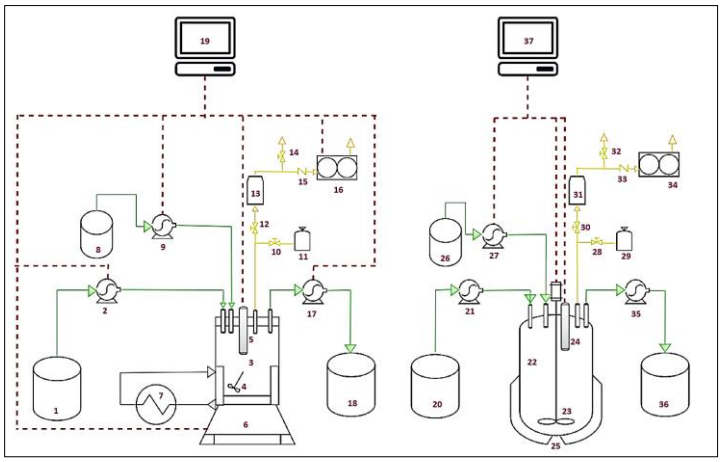


Fig. 10. Two stage system for hydrogen and methane production.

6.6 Nickel and iron addition to improve the biogas production in a two-stage system

This section corresponds to the specific objective 3. This work is intended to evaluate the effect of Fe^{2+} and Ni^{2+} addition on H_2-rich biogas production from organic solid waste and the CH_4-rich biogas production from the AE enriched with TM.

6.6.1 Determination of the Fe and Ni concentrations

Batch tests were carried out to measure the biohydrogen potential (BHP) according to the protocol established by Carrillo-Reyes *et al*., (2019) using the equipment automated methane potential test (AMPTS II; Bioprocess Control AB, Lund, Sweden). The temperature was set at 37±1°C and the initial pH was adjusted to 7.5±0.2. The substrate/inoculum rate (S/I) was 2.7 in terms of VS, and the substrate concentration was 15 gVS/L. The experiment was carried out for 30 hours. The effect of experimental variables (Fe^{2+} and Ni^{2+}) on HY and VS removal was evaluated with a central composite design (two factors). The results of the experimental variables were represented by a response surface methodology using design expert software (6.0.10). The Ni^{2+} concentrations tested were: 0, 0.08, 0.17, 0.25, 0.33, 0.42, and 0.5 mg/L. In the case of Fe^{2+}, the concentrations analyzed were 0, 111, 222, 334, 445, 556, and 667 mg/L. These concentrations were proposed based on a literature review where Fe^{2+} and Ni^{2+} were used to increase the H_2-rich biogas production (Karadag and Puhakka, 2010; Mullai *et al*., 2013a; Taherdanak *et al*., 2016b; Wang and Wan, 2008b). The configuration of the reactors was as follows for Fe^{2+} and Ni^{2+}, respectively (0 and 0, 0 and 0.25, 0 and 0.5, 333 and 0, 333 and 0.25, 333 and 0.5, 667 and 0, 667 and 0.25, 667 and 0.5, 730 and 0.25). Batch tests were performed in duplicate. Fe^{2+} and Ni^{2+} were added as $FeSO_4 \cdot 7H_2O$ and $NiCl_2 \cdot 6H_2O$, respectively.

6.6.2 Acidogenic digester (first stage)

An acidogenic sequencing batch reactor (SBR) with a working volume of 1 L and a headspace volume of 0.5 L was utilized for the first stage. The acidogenic reactor was equipped with a programmable logic controller (PLC) to control the pH, feed, and discharge. The emptying and filling time were 5 minutes, while the settling and sedimentation time were 50 minutes. The reactor operated with an OLR of 60 gVS/L·d and a hydraulic retention time (HRT) of 16 h. The temperature and pH were 37±0.5 °C and 5.5±0.2, respectively. The acidogenic SBR was operated for 32 cycles. The first 25 cycles were evaluated without TM addition to compare the results before and after the supplementation. From cycle 26, the TM were added according to the results obtained in the batch tests (0.25 mg/L of Ni^{2+} and 334 mg/L of Fe^{2+}). In cycles 37, 43, and 46-52, the TM were added again to determine the effect of the frequency of the supplementation on productivity and HY.

6.6.3 Methanogenic digester (second stage)

The second stage to produce CH_4-rich biogas was carried out in a sequencing batch reactor (SBR) in an INFORS HT model Labfors 5 reactor with a capacity of 3.2 L and a working volume of 2.8 L. The settling, discharging, and feeding times were 50, 5, and 7 minutes, respectively. The feeding and discharge were accomplished with peristaltic pumps Masterflex Model 77200-60 and 77200-50, respectively. The exchange volume was 500 mL of undiluted AE. The operational conditions were a stirring speed of 80 rpm, initial pH of 7.5±0.2, a temperature of 37±0.5 °C, and an HRT of 2.8 d. The inoculation was carried out with a S/I ratio of 2 in terms of VS. The reactor was operated for 10 cycles before the evaluation period to activate the sludge and achieve constant biogas production.

7 RESULTS AND DISCUSSION

This section includes the results of sections 1 and 2 of the methodology. Section 1 includes the study on the effect of nickel concentration on biohydrogen production: organic solid waste vs. glucose, and biogas production enhancement from OFMSW in a two stages system by nickel addition compared with a conventional digester. Section 2 contains the evaluation of nickel and iron addition to improve the biogas production in a two-stage system.

7.1 Effect of nickel concentration on biohydrogen production: organic solid waste vs. glucose

The batch tests using glucose as a reference substrate showed the highest H_2 production when 2 mg Ni^{2+}/g $VS_{inoculum}$ was applied (productivity and yield of 774±7.3 mL H_2/L/d and 55.8 ± 3.4 mL H_2/g of glucose respectively). H_2 production increased 34.4% compared to the control without nickel (Table 5). Similar H_2 production was obtained when concentrations of 1 and 5 mg Ni^{2+}/g $VS_{inoculum}$ were used (736 ± 2.5 and 743 ± 7.0 mL, respectively). One of the main differences between the treatments was the lag phase; the lowest lag phase corresponded to the control reactor, and the exponential phase began at 6.5 ± 0.1 hours. In the case of the reactors in which the highest concentrations of nickel were added, the lag phase was longer, approaching 8 h. To decrease the lag phase, it is necessary to preadapt the sludge to the concentrations of trace metals that will be used to activate the biocatalytic potential of microorganisms and enzymes (Chen *et al*., 2008; Ezebuiro *et al*., 2018).

Table 5. Hydrogen production, kinetic parameters, yields and carbohydrates removal at different initial-added nickel concentration.

Substrate	Nickel concentration ($mgNi^{2+}/gVS_{inoculum}$)	Productivity (mL H_2/L/d)	H_{max} (mL)	R_{max} (mL/h)	ʎ (h)	Yield (mL H_2/g)*	Total Carbohydrates Removal (%)
Glucose	0 (control)	576 ± 1.9	255.1 ± 9.0	38.3 ± 10.1	6.5 ± 0.1	41.5 ± 0.1	37 ± 8.9
	0.1	630 ± 4.5	265.6 ± 3.3	50.3 ± 5.4	8.4 ± 0.2	45.4 ± 0.3	38 ± 8.5
	0.5	617 ± 8.2	271.2 ± 1.2	62.8 ± 7.9	7.9 ± 0.2	44.5 ± 0.6	27 ± 8.1
	1	736 ± 2.5	287.3 ± 3.9	86.7 ± 3.2	7.3 ± 0.6	53.0 ± 0.1	15 ± 9.4
	1.5	680 ± 9.1	289.9 ± 6.2	74.2 ± 6.0	7.8 ± 0.1	49.1 ± 2.1	18 ± 6.3
	2	774 ± 7.3	309.5 ± 9.5	72.1 ± 3.1	6.7 ± 0.3	55.8 ± 3.4	19 ± 7.9
	5	743 ± 7.0	297.6 ± 7.7	60.1 ± 6.2	7.5 ± 0.3	47.0 ± 1.9	23 ± 4.9
OFMSW	0 (control)	433 ± 6.5	162.7 ± 5.1	47.0 ± 8.5	6.6 ± 0.1	31.2 ± 4.4	42 ± 19.5
	0.1	371 ± 13.4	134.9 ± 4.9	56.6 ± 0.5	7.6 ± 0.1	26.7 ± 4.0	46 ± 9.8
	0.5	268 ± 1.7	102.3 ± 4.7	39.5 ± 3.1	6.6 ± 2.0	19.0 ± 0.5	64 ± 4.8
	1	406 ± 8.3	146.7 ± 11.1	58.4 ± 7.8	7.8 ± 0.8	29.0 ± 2.4	45 ± 8.9
	1.5	382 ± 6.2	138.1 ± 8.2	61.3 ± 3.5	7.6 ± 0.7	27.5 ± 1.6	65 ± 16.9
	2	331 ± 13.4	119.3 ± 8.9	85.9 ± 6.5	8.7 ± 0.8	23.7 ± 2.0	48 ± 5.6
	5	313 ± 12.5	125.6 ± 10.4	29.4 ± 2.8	7.3 ± 0.3	24.3 ± 2.4	63 ± 10.5

Yield (mL H_2/g)*, g of glucose or g of OFMSW respectively.

Regarding HY, the control reactor obtained a yield of 41.5±0.1 mL H_2/g $glucose_{added}$. The highest HY (55.8 ± 3.4 mL H_2/g $glucose_{added}$) was obtained in the reactor with 2 mg Ni^{2+}/g $VS_{inoculum}$. The HY values obtained in the present work are lower than those reported by Taherdanak *et al*., (2016), who added

different $NiSO_4$ concentrations to batch reactors using glucose as substrate and found that the addition of Ni^{2+} ions at all concentrations tested drastically enhanced the biogas yield. When 50 mg/L of Ni^{2+} was added, the highest yield (680 mL/g VS_{added}) of biogas was obtained with an H_2 content of approximately 50% (74% higher than the control test). Wang and Wan, (2008) obtained a maximum hydrogen production potential of 288.6 mL, and the maximum HY of 296.1 mL/g glucose was obtained at a Ni^{2+} concentration of 0.1 mg/L, which is a higher yield than the HY achieved in this study and can be related not only to the effect of nickel but also to the origin of the inoculum or the operating conditions.

The highest total carbohydrate removal (38%) was reached with a concentration of 0.1 mg Ni^{2+}/g $VS_{inoculum}$. This removal was similar to the removal efficiency obtained in the control. Removal efficiencies decreased according to the increase in Ni^{2+} concentrations, agreeing with the report of Li and Fang, (2007). They tested different nickel concentrations in batch reactors using sucrose as substrate, obtaining complete degradation in the controls where Ni^{2+} was not added, whereas with increasing concentrations, the substrate degradation was adversely affected. The final pH of the reactors was recorded between 5.5 and 5.2. The results of the VFA analysis showed that butyrate concentrations ranged from 36 mg/L to 69 mg/L (Fig. 11A). Propionate was generated only in the control reactor. All the reactors showed high ethanol concentrations (between 676 and 905 mg/L), while acetate concentrations were in the range of 426 to 628 mg/L. The ethanol concentration increases according to the increase in the different nickel concentrations tested, which is similar to that reported by Wang and Wan, (2008) who obtained increases in the ethanol yield when testing concentrations between 1 and 5 mg/L of Ni^{2+}. Regarding acetic acid in the same study, the yields decreased with increasing Ni^{2+} concentrations from 0.01 to 0.05 mg/L and from 0.1 to 0.2 mg/L, and it was not detectable with further increasing Ni^{2+} concentration from 0.2 to 50 mg/L. In another study by Mu *et al*., (2006), 0.5 mg/L $NiCl_2$•$6H_2O$ was added to a reactor operated at 37 °C, obtaining an ethanol concentration of 917 mg/L at the end of fermentation; this value is in the range of ethanol concentrations obtained in the present study.

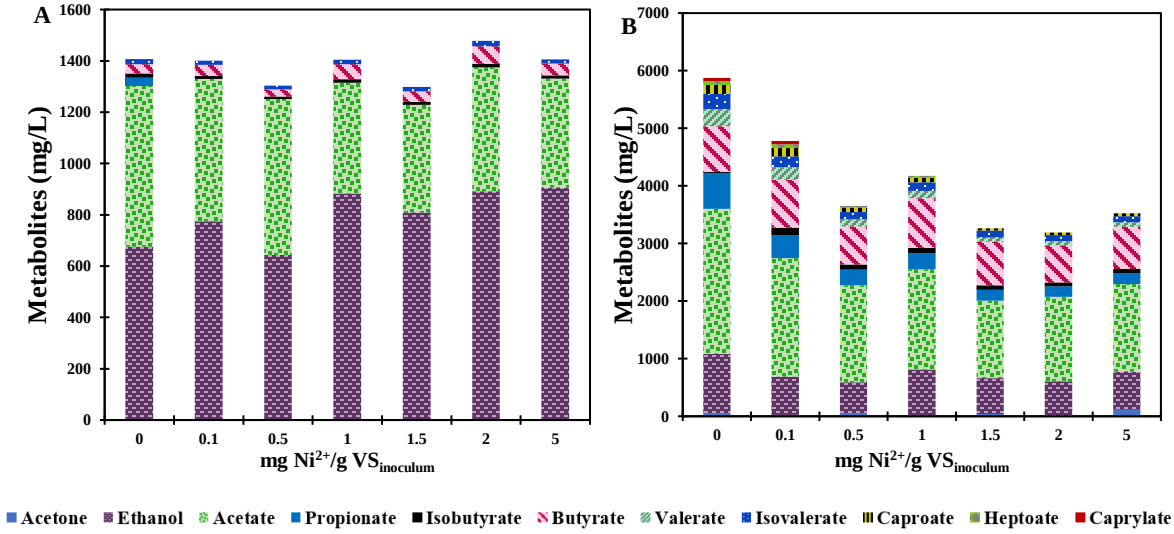


Fig. 11. VFA and metabolites generated during batch tests using A) glucose, and B) OFMSW as substrates.

OFMSW that contains fruits is one of the most widely used residues in the production of H_2 due to its high carbohydrate content (50–70% VS); when this type of residue is used, yields of up to 523 mL H_2/g VS_{added} have been achieved by using a mixture of fruits (Akinbomi and Taherzadeh, 2015), and up to 173 mL H_2/g VS_{added} has been achieved using vegetables. However, the composition of the substrate significantly affects the yields that can be obtained; for example, when using a carbon source such as cereals (rice, oats, and maize) as the substrate, yields of 96 mL H_2/g VS_{added} (Okamoto *et al*., 2000) or 134 mL H_2/g VS (Dong *et*

al., 2009) have been obtained. When different Ni^{2+} concentrations were applied to the reactors using OFMSW, the highest H_2 production was achieved in the control reactor with no nickel addition (Table 5). In general, the results were different for experiments where glucose was used as a substrate because OFMSW already contains an initial nickel concentration of 0.17 ± 0.06 mg Ni/g VS. The concentrations used in the reactors that presented lower H_2 production were 0.5, 2, and 5 mg Ni^{2+}/g $VS_{inoculum}$ (268 ± 1.7, 382 ± 6.2, and 313 ± 12.5 mLH_2/L/d, respectively); the highest production was obtained in the control (433 ± 6.5 mLH_2/L/d). Comparing the results with those obtained by Chen *et al*., (2021), who studied the effect of nickel on waste-activated sludge, a concentration of 5 mg/L of Ni^{2+} increased the accumulated H_2 volume by 29% compared with the control. However, the lag phase reported was at 20.4 h. In the present experiment, the lowest lag phase was obtained for the control (6.6 ± 0.1 h). The highest HY was also obtained in the control reactor (31.2 ± 4.4 mL H_2/gVS_{added}), while the lowest yield corresponded to the concentration of 0.5 mg Ni^{2+}/g $VS_{inoculum}$ (19 ± 0.5 mL H_2/gVS_{added}). HY did not increase as the concentrations of Ni^{2+} increased, agreeing with reports where easy-to-degrade substrates were tested (Li and Fang, 2007b; Lin and Lay, 2005b; Mullai *et al*., 2013b; Taherdanak *et al*., 2016b; Wang and Wan, 2008a). The OFMSW used in the present study had a Ni concentration of 0.17 ± 0.06 mg Ni/g VS, and the inoculum had a concentration of 0.0018 mg Ni/g $VS_{inoculum}$, avoiding Ni limitations.

pH plays a vital role in regulating metabolite synthesis pathways and microbial community structure, and it can influence the rate of hydrolysis and the production of VFA. Hence, the accumulation of soluble acid metabolites can lead to a sharp decrease in the pH system, inhibiting HY and leading to a low conversion rate (Akinbomi and Taherzadeh, 2015; Mu *et al*., 2006). Various values have been reported for OFMSW, with a predominance of pH 5.5 (Moreno-Andrade *et al*., 2015; Santiago *et al*., 2020; Wang *et al*., 2020), although the operation has also been carried out at pH values between 4–4.6 (Han *et al*., 2017). Due to VFA production, the pH decreased from 7.5±0.2 to 5.3-5.5, as usual in DF. These values were close to the optimum pH, which is 5-5.5 (Jun *et al*., 2008; Tang *et al*., 2022b). In addition to the above, considering the production of secondary metabolites and biogas, it is possible that the process has not been affected by this parameter. The highest total carbohydrate removal was 65±16.9% in the reactor with 1.5 mg Ni^{2+}/g $VS_{inoculum}$. The quantification of VFA demonstrated the generation of metabolites, such as acetone, butanol, caproate, heptanoate, and caprylate, that were not detected in the experiments with glucose (Fig. 11B). The control showed the highest metabolite concentration, which decreased when the Ni^{2+} concentration increased. This agrees with the results reported by Chen *et al*., (2021), who obtained the highest concentration of metabolites in the control and lower concentrations in the reactors with higher Ni^{2+} doses (50, 100, and 500 mg/L). ANOVA demonstrated that the interaction of the different factors was significant (P value <0.001); therefore, there was a significant difference between the interaction of the type of substrate and the concentration of Ni^{2+} added regarding YH_2 (Fig. 12).

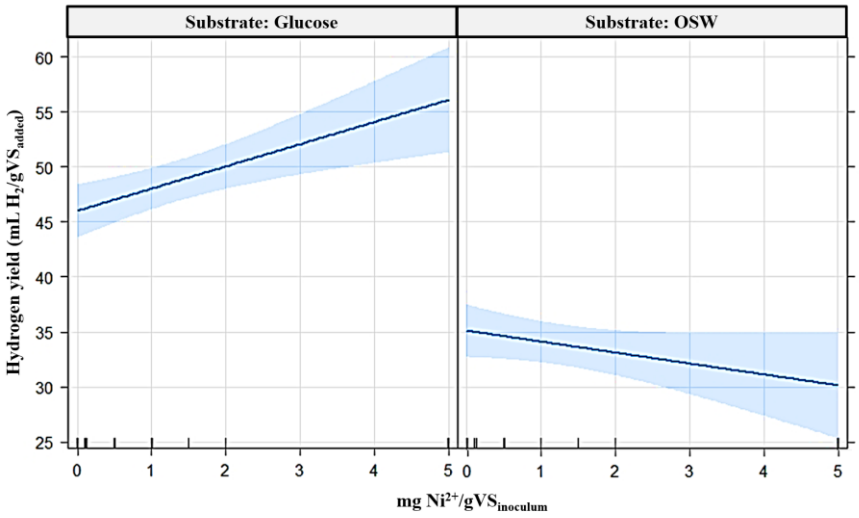


Fig. 12. Plot of concentration predictor effect of Ni^{2+} on YH_2: A) glucose and B) OFMSW.

7.1.1 Operation of the Sequencing Batch Reactor

Fig. 13A shows the H_2 production and HY for experiments without Ni^{2+} (as control) as well as 0.1 and 0.5 mgNi^{2+}/g $VS_{inoculum}$. The highest H_2 production (1501 ± 114 mL H_2) and HY (74.5 ± 12.7 mL H_2/g VS_{added}) corresponded to the cycles where Ni^{2+} was not added. The H_2 production decreased with the addition of Ni. The operation in the SBR presented a similar trend to the batch operation, where the highest H_2 production and HY were achieved without nickel addition.

The performances of the reactor during SBR and batch operation using OFMSW as substrate may be related to possible inhibition of the process due to Ni accumulation. In this regard, different factors can inhibit the DF process (Chen *et al.*, 2021), such as inhibitors in the mixed microflora; inhibitors from substrate pretreatment; inhibitors in-process, for instance, the accumulation of ammonia; H_2 partial pressure; and end-products, such as acetic acid/acetate, butyric acid/butyrate, propionic acid/propionate, formic acid/formate, and ethanol (Bundhoo and Mohee, 2016). Finally, the metal ions included iron, nickel, copper, manganese, zinc, chromium, cadmium, and lead. Although TE has been shown to limit the growth of microorganisms in terms of cell density (Choong *et al.*, 2016), excess Ni causes inhibition in intermediate processes of anaerobic digestion (Demirel and Scherer, 2011). The concentrations of TE that must be added to the reactors may vary according to the type of reactor, substrate, inoculum, etc. It has been reported that the minimum requirements for fermentation based on the active volume of the reactor are 200 mg Ni/m^3/d (Takashima *et al.*, 1990). However, Ni may accumulate in the system, causing inhibition, which may be the reason for the low production of biogas in the present study.

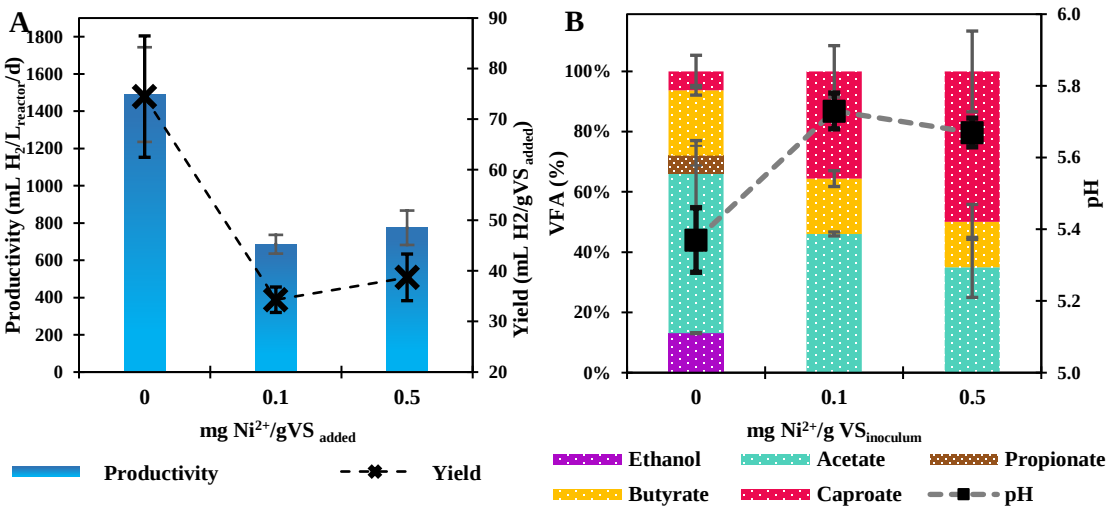


Fig. 13. A) Hydrogen productivity and yields, and B) generation of metabolites and pH during operation in SBR applying different initial-added nickel concentrations.

The final concentration of Ni in the digestate was analyzed in each of the conditions tested (in the control and after the addition of 0.1 and 0.5 mg Ni^{2+}/g $VS_{inoculum}$), resulting in 0.129, 0.801, and 1.23 mg/L of Ni, respectively, suggesting that nickel accumulates in the reactor after each addition, increasing the concentration in the digestate. However, this digestate could be recirculated in the system or used for methane production in a two-stage system since it has been reported that this trace metal has a vital role in anaerobic digestion in the growth of all methanogens and the synthesis of cofactor F_{430} (Aghajani Delavar and Wang, 2021; Dong *et al*., 2009; Okamoto *et al*., 2000). This coenzyme (F_{430}) is contained within the Methylcoenzyme M reductase enzyme, which reduces methyl coenzyme M to methane in all methanogenic pathways (Garuti *et al*., 2018; Glass and Orphan, 2012; Thanh *et al*., 2016b).

Fig. 13B shows the production of the metabolites at different Ni concentrations. Acetate, butyrate, and caproate were present in all the conditions. When nickel was added, the production of ethanol and propionate was no longer detected. The average concentrations of acetate and butyrate decreased according to the increase in added nickel concentrations. Likewise, the caproate concentration increased according to the increase in the nickel concentration. At 0.5 mg Ni^{2+}/g $VS_{inoculum}$ caproate was the main metabolite produced. In our investigation, a thermally pretreated inoculum was used; in this way, cultures of H_2-producing bacteria are usually dominated by clostridial species (Maintinguer *et al*., 2008). The HY decrease reflects changes in the metabolism and composition of microbial communities. Consequently, different subproducts are obtained due to the flexibility of the metabolism of microorganisms, e.g., in the clostridial species (Quéméneur *et al*., 2011). Some clostridial species can switch their metabolism from the production of H_2, acetate, and butyrate to solvent production (e.g., ethanol) depending on the operating conditions (Janssen *et al*., 2010; Schaffer *et al*., 2002). Therefore, by decreasing the acetate and butyrate concentrations, it is possible that the reverse β-oxidation pathway had benefited, in which chain elongation of VFA to medium-chain fatty acids (MCFA), as caproate, was carried out using an electron donor such as ethanol (De Vrieze *et al*., 2015; Steinbusch *et al*., 2011; Roghair *et al*., 2018; Thauer *et al*., 2010; Vignais and Billoud, 2007). Also, it is possible to produce caproic acid through the sequential chain elongation step, according to reactions (Eq. 2 and 3) mentioned by Piffer *et al*., (2021). Although lactic acid was not measured during the reactor´s operation, there are reports that it can be generated by the DF process (Ahmad *et al*., 2022; Demichelis *et al*., 2017) and used with the butyrate to generate caproic acid (Eq. 3).

Acetic acid + 2 Lactic acid → H_2 + 1.5 Butyric acid + 2 CO_2 + H_2O (Eq. 2)

$\Delta G^{\circ\prime}$= - 156.6 kJ/mol

Lactic acid + Butyric acid → Caproic acid + H_2O + CO_2 (Eq. 3)

$\Delta G^{\circ\prime}$= - 57.2 kJ/mol

The [NiFe]-hydrogenase catalyzes the reversible reaction for H_2 production. However, it has been reported the influence of the enzyme in H_2 consumption (Vignais and Billoud, 2007). Therefore, the low HY in the last stage of the reactor´s operation could be explained by hydrogen consumption. Also, the prevalence of caproate as the main metabolite over acetate and butyrate, when the dose of 0.5 mgNi^{2+}/g$VS_{inoculum}$ was applied, indicates that different metabolic pathways were expressed during the last experimental stage, reducing the HY. When 0.1 and 0.5 mg Ni^{2+}/g $VS_{inoculum}$ were added to OFMSW, no ethanol was detected in the digestate. However, in the batch tests where glucose was used as a substrate, it was noted that ethanol concentrations increased according to the increase in Ni^{2+} concentrations. Therefore, it is possible that the ethanol generated was used as an electron donor for chain elongation. Our results agree with those obtained by Ashley *et al.*, (1982), who described an increase in VFA and MCFA with increasing nickel concentrations in an anaerobic digestor. Food waste has also been reported as an electron acceptor substrate for MCFA production. Roghair *et al*., (2018) used hydrolyzed and acidified food waste and additional ethanol to develop a continuous chain elongation process, obtaining up to 5.5 g/L/d of n-caproate at a hydraulic retention time of 4 d. Similarly, Reddy *et al*., (2018) employed a two-stage mixed culture fermentation to produce short-chain fatty acids and MCFAs using food waste as the electron acceptor, obtaining up to 8.1 g/L of caproic acid and 8.9 g/L of butyric acid using a bioaugmented culture. However, it is necessary to elucidate the role of Ni^{2+} in the stimulation of the chain elongation process to obtain higher MCFA productivity.

7.2 Biogas production enhancement from OFMSW in a two stages system by nickel addition compared with a conventional digester

The effect of Ni^{2+} in the acetogenic and methanogenic stages is well known, while its effect in the hydrolysis and acidogenesis stages is still under study. In this sense, the main objective of this section was to determine the effect of the addition of different concentrations of Ni^{2+} on the production of H_2-rich biogas through DF process from OFMSW followed by CH_4-rich biogas production in a second stage. We also explored the impact of TSAD and OSAD as well as the addition of TM on microbial communities to analyze potential microbiological drivers of AD performance.

7.2.1 Conventional anaerobic digestor (a single stage)

The OSAD evaluation began with an OLR of 1.5 gVS/L·d for four cycles after the acclimatization period (Fig. 14). The results of the biogas production in this period were productivity of 170±40 mL CH_4/L·d and a specific methane yield (SMY) of 51±9.3 mL CH_4/gVS_{added}. Subsequently, the OLR was increased to 2.5 gVS/L·d from cycles 5 to 14. In this period, both the productivity and the SMY increased to 373±71 mL CH_4/L·d and 76±15 mL CH_4/gVS_{added}, respectively. However, these values were lower than the productivities reported by Voelklein *et al*., (2017) who obtained a SMY of 324.5±25 mL CH_4/gVS with an OLR of 2 gVS/L·d. The values obtained are also lower than those obtained by Jiménez-Ocampo *et al*., (2021), who achieved a SMY and productivity of 87 mL CH_4/gVS_{added} and 487 mL CH_4/L·d respectively. However, the substrate used in this study is more complex since it is an actual sample of OFMSW that contains traces of non-degradable materials, explaining the lower production of biogas.

The removal of TS and VS during cycles 1 to 4 with the first OLR evaluated were 42.9±14.6% and 48.7±14.1%, respectively. When the second OLR (2.5 gVS/L·d) was tested, the removals of TS and VS were 50.5±4.2% and 63.7±15.1%, respectively. These results indicated that the reactor was stable. The VS degradation was similar to the values obtained by Aramrueang *et al*., (2016) (53 and 66% with an HRT of 15 d) who indicated that a high HRT contributes to greater VS degradation and higher SMY. Since the reactor had an automatic control system for the pH, no variations were detected (7.5±0.2); this value is in the optimum pH range for the methanogenic stage (Zamri *et al*., 2021).

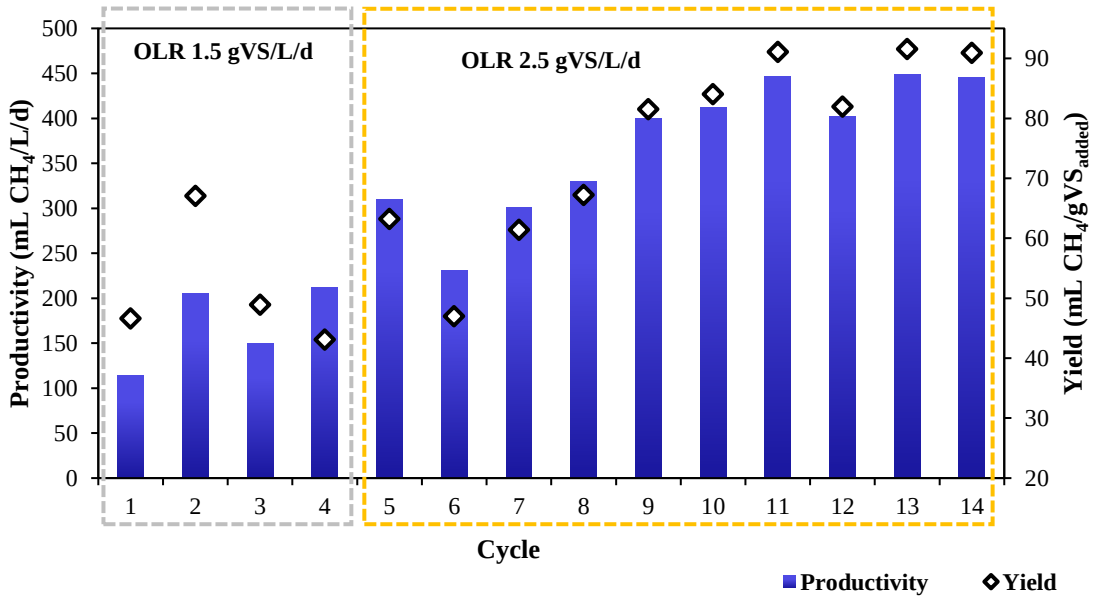


Fig. 14. Productivity and yields of methane during operation of a conventional anaerobic digestor (single stage).

7.2.2 Acidogenic digester (first stage)

The operation of the acidogenic reactor was carried out under three different conditions to evaluate the effect of Ni^{2+} amendments (Fig. 15). The OLR and the HRT were kept constant for all the cycles. Application of 0, 0.1, and 0.5 mg $Ni^{2+}/gVS_{inoculum}$ led to productivities of 1809±361, 1670±338, and 2738±199 mL H_2/L·d, respectively. The hydrogen yield (HY) obtained for each condition was 30±6, 28±5.6, and 46±3 mL H_2/gVS_{added}. The average of the productivities when the highest Ni^{2+} concentration was applied from cycle 15 to cycle 32 was 2738±199 mL H_2/L·d (Table 6), reaching the highest value in cycle 20 with a productivity of 3089 mL H_2/L·d, which is similar to those productivities achieved by (Angeriz-Campoy *et al*., 2018, 2017, 2015) who obtained 3.33, 3.67 and 2.51 L H_2/L·d which are the highest productivities reported in the literature for this type of substrate. The reactor operated stably for 32 cycles.

Different Ni^{2+} concentrations have been used in DF and AD processes to increase biogas production. In the case of H_2 production through the DF, Taherdanak *et al*., (2016) used 0.25, 0.5, 2.5, 5, 10, 25, and 50 mg Ni^{2+}/L in batch reactors using glucose as substrate; the best HY were obtained with the concentrations of 10 and 25 mg Ni^{2+}/L (350 and 380 mL H_2/gVS), respectively. Similarly, Wang and Wan (2008) operated batch reactors using glucose as substrate, to test the concentrations 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 5, 10, 20, 50 mg/L of Ni^{2+}, they obtained the highest HY of 296 mL H_2/g of glucose by applying 0.1 mg Ni^{2+}/L. Due to the different types of substrates that have been used to prove the Ni^{2+} effect on H_2 production, a fair comparison is not possible since the substrate, inoculum, and operational conditions influence the results. Nevertheless, Chen *et al*., (2021) used a complex substrate (activated sludge) to operate batch digesters using the Ni^{2+} concentrations of 0, 5, 10, 50, 100, and 500 mg Ni^{2+}/L; the highest HY of 480 mL H_2/g COD was obtained by applying 5 mg Ni^{2+}/L. According to the results obtained in this study, it was possible to achieve high productivity and maintain stability in the DF process by applying 0.5 mg $Ni^{2+}/gVS_{inoculum}$, since no signs of inhibition were detected even when the feedstock is a complex substrate. The increase in productivity by adding 0.5 mg $Ni^{2+}/gVS_{inoculum}$, is similar to the results obtained by Gou *et al*., (2015) who obtained a HY of 2.05 mol H_2/mol sucrose by applying 0.6 mg/L of Ni^{2+}, which corresponded to two folds in the yield obtained in the control.

Table 6. Results of the operation of the acidogenic reactor (first stage).

Parameter	Units	Cycles (1-6)	Cycles (7-14)	Cycles (15-32)
Ni^{2+} addition	$mg/gVS_{inocumum}$	0	0.1	0.5
HRT	d	0.67	0.67	0.67
OLR	gVS/L/d	60	60	60
pH	-	6.1±0.1	6.3±0.07	6.2±0.1
Total solids	g/L	56.3±10.6	40.0±13.1	40.5±11.1
Volatile solids	g/L	25.4±3.9	17.8±4.8	17.3±3.6
COD	g/L	43.1±1.6	41.9±1.7	38.9±3.5
Total VFA	g/L	6.4±0.3	6.1±1.7	9.0±2.4
YH_2	mL H_2/g VS added	30±6	28±6	46±3
Productivity	mL H_2/L/d	1809±361	1670±338	2738±199

The addition of the different Ni^{2+} concentrations improved the removal of TS, VS, and COD. On average, the remotion of TS, VS, and COD for the operating cycles 1 to 6 was 42.5±10.8%, 41.1±9%, and 26.6±0.3, respectively. For the following cycles (7-14) the removals for TS, VS, and COD were 59.1±13.4%, 58.7±11%, and 28.5±3%, respectively. During the last operating cycles from 15 to 32, the highest solids remotion was observed, and it was more constant than in the previous cycles. The average removal for TS, VS, and COD was 64.3±0.9%, 64±0.7%, and 33.6±0.6%, respectively.

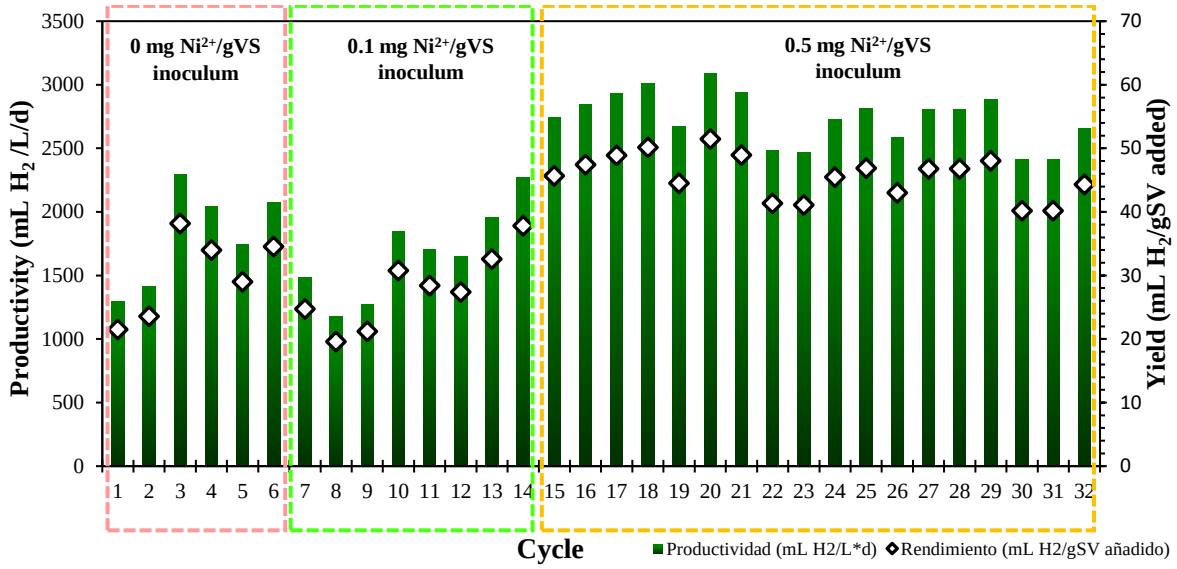


Fig. 15. Productivity and yields of hydrogen during operation at SBR (First stage)

The VFA concentrations were measured in the acidogenic effluents. Important changes were observed mainly in the last cycles (Fig. 16). During the first cycles of operation without Ni^{2+} addition (1-6), the total production of metabolites was 6358±334 mg/L, with 42.6% corresponding to acetate. In all cases, acetate was the VFA generated at the highest concentration. Although it has been reported that acetate and butyrate are the VFA generated at the highest concentration during the DF process (Angeriz-Campoy *et al*., 2017), caproate concentrations were slightly higher than butyrate. In the cycles of operation performed without Ni^{2+} addition (1-6), the production of caproate corresponded to 24.7% while the production of butyrate was 24% VFA. In the following cycles (7-14) where the concentration of 0.1 mgNi^{2+}/$gVS_{inoculum}$ was supplied, the VFA concentration increased by 33.6% and subsequently decreased, obtaining a metabolite concentration of 4941 mg/L. The concentration of total VFA in this study exceeds those obtained by Chen *et al*., (2021) who reported a concentration of 973 mg VFA/L in the control and lower concentrations of total VFA when applying the different doses of Ni^{2+} (5, 10, 50, 100 and 500 mg/L), in each case, the acetate concentration was higher than the butyrate concentration.

In the following cycles from 15 to 32, the concentration of 0.5 mg Ni^{2+}/$gVS_{inoculum}$ was added. The VFA concentrations were similar to those obtained in the second condition. However, after cycle 21 an increase of 73.1% in the total metabolites' concentration was observed. Acetate was the VFA produced in the highest concentration reaching 4031±99.2 mg/L. The caproate concentrations measured in the acidogenic effluent were 2520±218 mg/L. In the last cycles of operation and after the Ni^{2+} supplementation, a concentration of 1402±645 mg/L of caprylate was detected, corresponding to a medium-chain fatty acid (MCFA). Caprylate was generated after long-term reactor operation. Therefore, the production of bio-H_2 improved with the Ni^{2+} addition as well as the production of the metabolites. The generation of higher molecular weight metabolites due to the addition of nickel in the AD process was reported by Ashley *et al*., (1982), who indicated that the presence of Ni^{2+} can shift the emphasis of fermentation towards that of amino acids and away from carbohydrate metabolism.

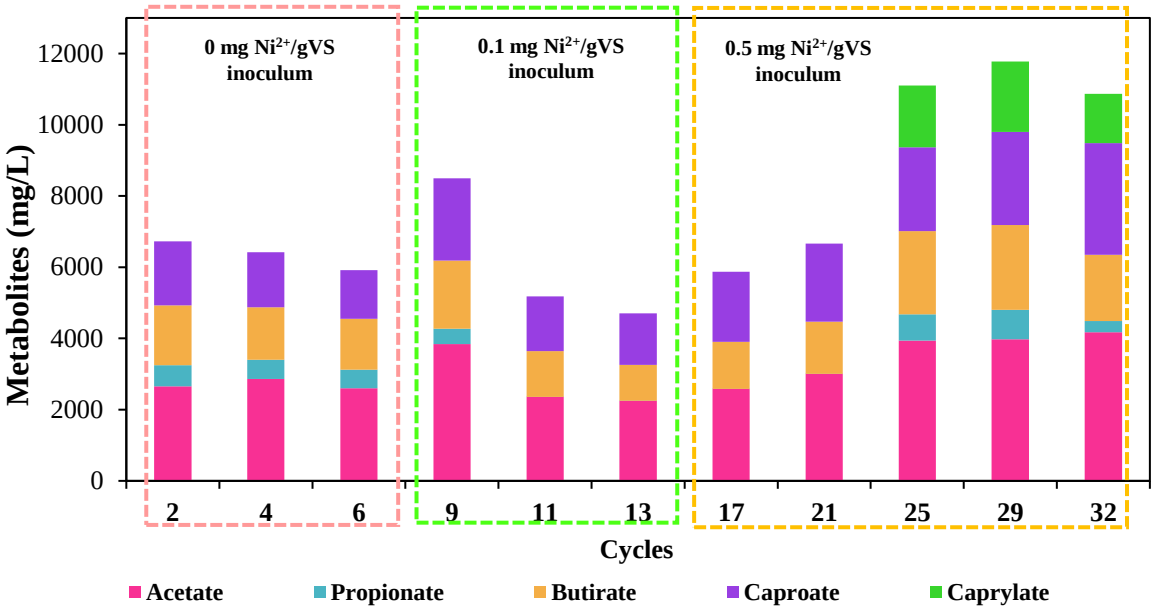


Fig. 16. Generation of VFA during operation in SBR (first stage)

7.2.3 Biochemical methane potential assay

Triplicate tests were carried out to analyze the BMP of the three types of effluents generated during the operation of the acidogenic reactor (Fig. 17). The highest production of CH_4 was reached with the effluent without Ni^{2+}, with a productivity of 2600±55 mL CH_4 and a SMY of 173±5 mL CH_4/gVS_{added}. The effluent from cycles 7 to 14 of the acidogenic reactor (with a Ni^{2+} supplementation of 0.1 mg $Ni^{2+}/gVS_{inoculum}$) presented a production of 2054±53 mL of CH_4 and a yield of 137±4 mL CH_4/gVS_{added}. The lowest production and SMY were obtained with the acidogenic effluent obtained from cycles 15 to 32; the CH_4 production reached 1669±32 mL of CH_4 and a yield of 112±2 mL CH_4/gVS_{added}. It is noteworthy to mention that the batch tests inoculated with not acclimatized granular sludge as inoculum to treat acidogenic effluents enriched with Ni^{2+}. This is a factor that could have affected the test since the effluents enriched with nickel can produce toxic effects on the microorganisms (Demirel and Scherer, 2011; Ezebuiro and Körner, 2017).

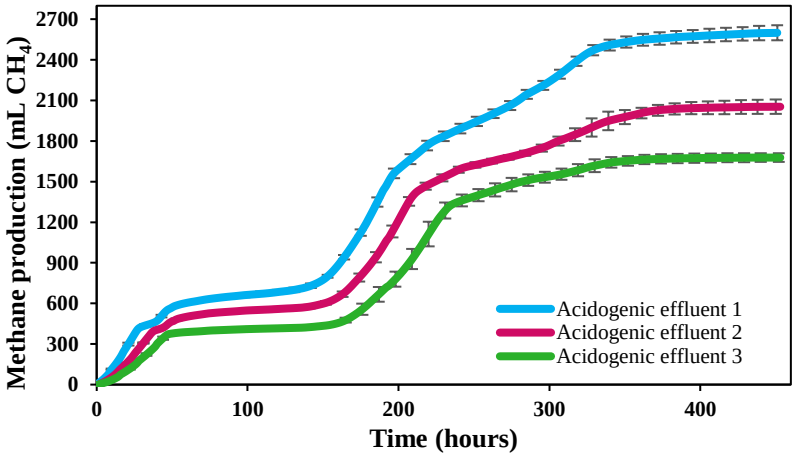


Fig. 17. Cumulative volume of methane produced in batch tests from acidogenic effluents nickel enriched.

7.2.4 Methanogenic digester (second stage)

The effluents from each operational condition of the acidogenic reactor were homogenized and used as substrates to feed the methanogenic reactor. In this stage, no dilutions were carried out. In cycles 1 to 4 when the first acidogenic effluent was fed, a productivity of 641±95 mL CH_4/L·d and a SMY of 156±23 mL CH_4/gVS_{added} were obtained (Fig. 18). Both the productivity and SMY were higher than those obtained when the reactor operated in one stage. This agrees with the assessments of Ezebuiro and Körner, (2017) who concluded that a two-stage operation is better to obtain higher productivity and SMY since a hydrolyzed effluent rich in VFA, mainly acetate, is used as a substrate, which can be utilized by methanogenic microorganisms for CH_4 production. The SMY based on the VS amount fed in the first stage, in cycles 1 to 4 is 138±21 mL of CH_4/gVS. This value is lower than the SMY obtained from the AE since the AE contain a part of hydrolyzed organic matter that cannot be determined as VS. During the operation of the reactor in cycles 5 to 8, where the second effluent was used as feedstock, the reactor achieved productivities and SMY of 657±132 mL CH_4/L·d and 216±44 mL CH_4/gVS_{added}, respectively. These values were slightly higher than those obtained with the first effluent tested. However, the low production could be explained due to the change in substrate since its composition in terms of VFA concentrations was different and it contained a higher Ni concentration than the first effluent.

Once the third effluent was fed from cycle 9, the productivity increased up to 798±69 mL CH_4/L·d, corresponding to an increase of 22.4% concerning the values obtained when the first digestate was used. In cycles 9 to 11, the SMY was 241±8 mL CH_4/gVS_{added}; however, in the operating cycles from 12 to 15, the SMY increased to 365±20 mL CH_4/gVS_{added}, which corresponds to an increase of 127% compared with the first condition tested in the TSAD. This result reaffirms that the use of TM as Ni^{2+} can increase SMY. Comparing the productivities obtained in OSAD with TSAD, it was possible to increase productivity and SMY by 72% and 105%, respectively by operating in TSAD (using the effluent without Ni^{2+} supplementation). Regarding the Ni^{2+} effect, the CH_4-productivity and the SMY increased 127% and 380%, respectively, when the reactor was fed with the effluent enriched with Ni^{2+} (from cycles 15-32 of the acidogenic reactor).

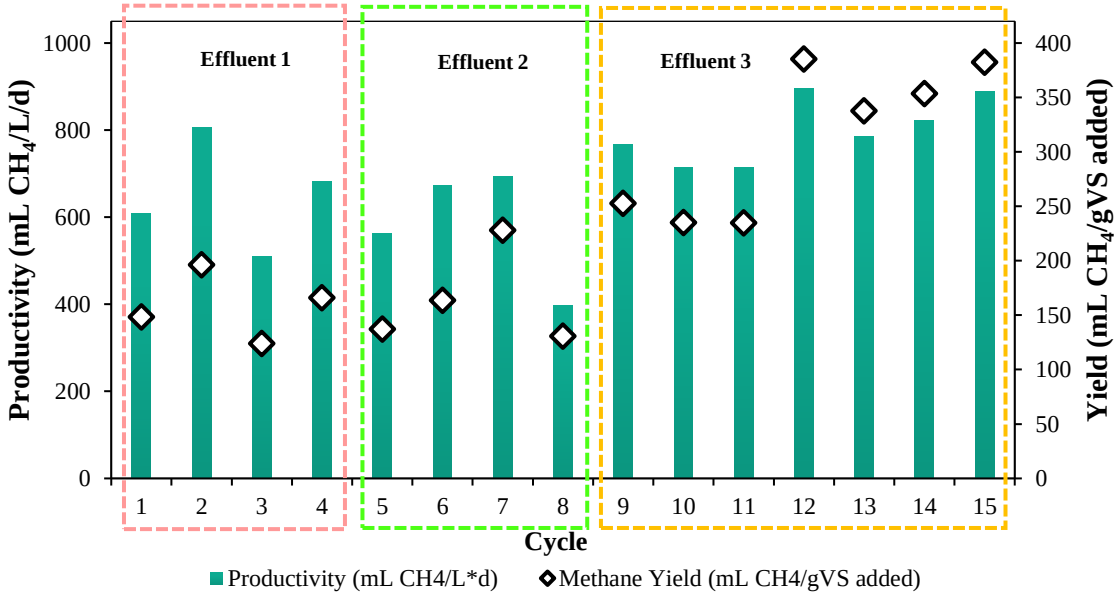


Fig. 18. Productivity and yields of methane during operation at SBR (second stage).

Similarly, as the acidogenic reactor, the removal of TS and VS increased in each condition tested in the second stage. For cycles 1-4, TS and VS removal were 11.3±1.8% and 18.9±4%, respectively. For the

operating cycles from 5 to 8, the TS and VS removal were 14.5±0.9% and 21.7%, respectively. In the last cycles, the highest TS and VS removals were reached, corresponding to 21.6±1.7% and 25.8±3.2%, respectively (Table 7). The removal of solids and COD were also reported by Azizi *et al*., (2019), who operated a TSAD system from source-separated organics from OFMSW and increased the degradation kinetics up to 45% through thermal pretreatment. Due to the capacity of the two-stage system to remove TS, VS, and COD, it was possible to obtain a digestate with a lower content of organic matter, which could have agricultural applications (Peng *et al*., 2020).

Table 7. Results of the operation of the methanogenic reactor (Second stage).

Parameter	Units	Cycles (1-4)	Cycles (5-8)	Cycles (9-15)
Ni	mg/gTS	1.67	1.85	2.24
OLR	gVS/L·d	1.97	1.38	1.34
HRT	d	13	13	13
pH	-	7.8±0.3	7.7±0.2	7.7±0.3
TS	g/L	49.9±1.0	34.2±0.4	31.7±0.7
VS	g/L	20.6±1.1	13.9±0.4	12.8±0.6
COD	g/L	14.5±1.4	10.6±1.9	9.2±1.2
Total VFA	mg/L	Not detected	Not detected	Not detected
SMY (AE)	mL CH_4/g VS_{added}	156±23	216±44	365±20
Productivity	mL CH_4/L·d	641±95	657±132	847±46

Regarding the VFA, in the three conditions tested during the TSAD, VFA were not detected at the end of each cycle of operation, which reinforces what has been described by several authors on the use of TM to promote VFA degradation. For instance, Espinosa *et al*., (1995) achieved a propionic acid reduction from 5,291 mg/L to 251 mg/L and an acetic acid reduction from 1,100 mg/L to 158 mg/L in a UASB reactor using a mix of Fe, Co, Mo, and Ni. In the same way, Osuna *et al*., (2003) induced the propionate degradation in an Up-flow anaerobic sludge bed reactor by the addition of a TM solution containing Fe, B, Zn, Mn, Cu, Ni, and Se, and Wall *et al*., (2014), obtained low concentrations of propionic acid in a CSTR by adding Co, Fe, and Ni. In the aforementioned cases, certain Ni^{2+} concentrations were added to the AD process.

7.2.5 Microbial community analysis

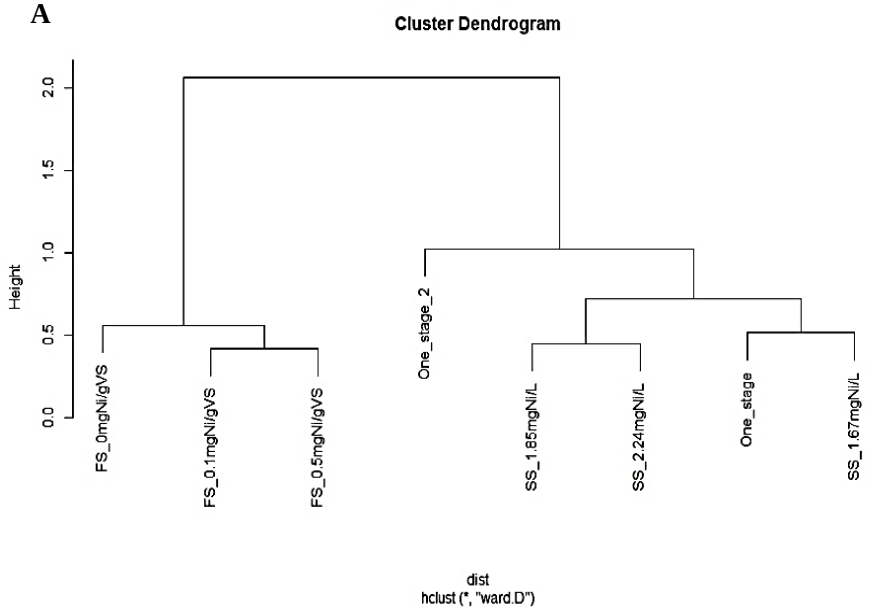
AD involves different microorganisms (bacteria and archaea) that degrade organic matter and generate methane-rich biogas. According to the results obtained from all the samples analyzed, a cluster dendrogram was generated (Fig. 19A), it shows a variety of clustering patterns indicating important differences in community structure in samples from each stage, the first group corresponds to the acidogenic stage, and the second group corresponds to the methanogenic stages. The samples of the OSAD were cluster separated. At the beginning of the operation of the OSAD, the microbial community revealed a high relative abundance of Bacteroidota reaching 60%, and similar abundances of Thermotogota (11%), Sinergistota (11%), and Firmicutes (11%) (Fig. 19B).

After 10 cycles, the microbial community shifted and the relative abundance of Bacteroidota dropped to 10%. This behavior was also observed by Fan *et al*., (2022) who studied the microbial communities changes in UASB reactors; in that case the phyla Bacteroidota dropped from 22% to 16% which was related to the poor efficiency of acetate metabolization. At the end of the operation of OSAD, the main phyla were Firmicutes (20%), Synergiostota (20%), Thermotogota (8%), and other phyla with a relative abundance lower than 5% (Bacteroidota, Caldatribacteriota, Cloroflexi, Cloacimonadota, Desulfobacterota, Euryarchaeota, and Verrucomicrobiota); thus, microbial communities changed along the time. According to Tao *et al*., (2020), Bacteroides is a versatile taxon present in anaerobic reactors and plays an important role in hydrolyzing a large variety of polysaccharides and producing VFA;

nevertheless, this taxon was displaced by Firmicutes and Synergistota. In AD, Firmicutes plays an important role in metabolizing organic compounds and producing VFA (Strazzera *et al*., 2021); meanwhile, *Synergistacea* (Synergistota phyla) can produce H_2 and CO_2 from the degradation of monocarboxylic and long-chain fatty acids, which could implicate a syntropic association with hydrogenotrophic methanogens (Hardy *et al*., 2021).

Regarding the first stage of the TSAD, H_2-rich biogas production through DF requires sludge pretreatment to avoid CH_4 production. In this case, a pretreated sludge was used for the inoculation of the acidogenic reactor in the first stage. Nevertheless, this kind of pretreatment changes the microbial communities since, a heat shock pretreatment causes the suppression of methanogenic Archaea and non-sporulating bacteria, enriching the culture with sporulating H_2-producing bacteria such as Clostridia (Lay *et al*., 2003). At the beginning of the operation, around 70% of the relative abundance corresponds to Firmicutes phylum with a high abundance of Clostridia (50%) and Bacilli (20%) class. These results coincide with the microbial communities reported by O-Thong *et al*., (2009), who described a high abundance of Firmicutes phyla, clostridia class, mainly *Thermoanaerobacterium thermosaccharolyticum* after the thermal pretreatment, which corresponds to a spore-forming bacteria. According to Yang and Wang, (2019), it is possible that Firmicutes phyla had a better ability to recover their activity after a heat-shock pretreatment compared with other phyla. This is beneficial in the acidogenic stage since Firmicutes had the ability of degrading macromolecules such as proteins, lipids, and polymeric carbohydrates, and produce VFA (Ko *et al*., 2018).

After the Ni^{2+} addition (0.1 mg $Ni^{2+}/gVS_{inoculum}$), from cycles 7 to 14, changes in microbial communities were detected. The relative abundance of Firmicutes phylum dropped from 70% to 60% and an increase in Bacteroidota was detected from 13% to 23%. Also, the relative abundance of Actinobacteriota decreased; this phylum had been detected in anaerobic digesters and is related to the degradation of recalcitrant organic matter and hydrolysis of complex carbohydrates (Theuerl *et al*., 2020). After the second period of Ni^{2+} addition (0.5 mg $Ni^{2+}/gVS_{inoculum}$) from cycle 15 to 32, the phylum Actinobacteriota increased from 5% to 12%, which could be related to the period of adaptation to the Ni^{2+} in the medium.



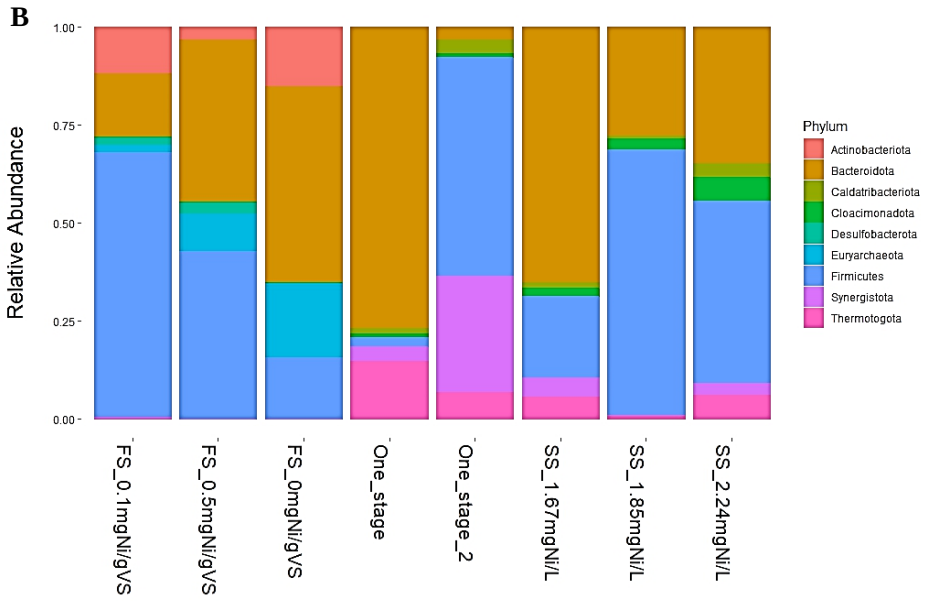


Fig. 19. Cluster dendrogram Based on Bray-Curtis distance (A) and relative abundance(B) of microbial communities at phylum level for SBR in different stages and Ni concentrations. (FS=first stage, SS=second stage)

Regarding metabolites production, caproate production was observed in all the cycles of the acidogenic reactor, and it was one of the principal metabolites produced in this stage besides acetate and butyrate. Through chain elongation process, bacteria transform ethanol and short-chain fatty acids such as acetate and butyrate into medium-chain fatty acids (MCFA) (Agler *et al*., 2012). Wallace *et al*., (2003) reported that *Eubacterium pyruvativorans* sp., isolated from sheep rumen fluid was able to produce caproate and valerate by acetate and propionate utilization. In this case, Eubacteriaceae family (Firmicutes) was found in the samples of the first stage operation, which could be related to the MCFA production since chain elongation can be performed under non-sterile conditions with a mixed microbial culture (Reddy *et al*., 2018) as the one used to inoculate the acidogenic reactor.

The composition of microbial communities were constrained against physicochemical variables in a dbRDA where the first and second axes explained 56.94% and 22.86% variation of microbial communities (ANOVA, p=0.038). The COD removal was correlated with Ercella succinigenes and Thermovirga sp. (Firmicutes and Synergistota phyla respectively). The Ni^{2+} addition was correlated with *Syntrophomonas* sp., and *DMER64* sp. (Firmicutes and Bacteroidota phyla, respectively), and the productivity of the first stage was correlated with the genus *Prevotella*_7 sp., and *Pseudoramibacter* sp., (Bacteroidota and Firmicutes phyla, respectively). These phyla were predominant in each stage evaluated. Cloacimonadia and JS1 were the most significant class in the first stage, and Methanobacteria and Actinobacteria were the predominant class in the second stage. Meanwhile, Fig. 20 showed that genus *LNR A2-18* was the most significant in the first stage, and *Methanobrevibacter* and *Methanosphaera* were the predominant genera in the second stage.

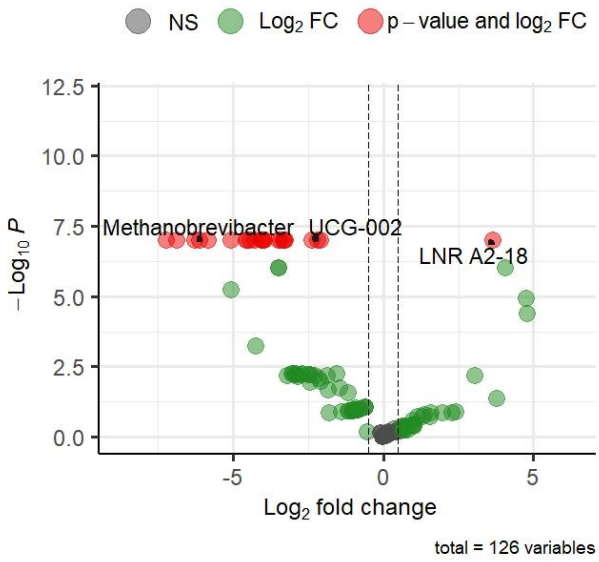


Fig. 20. Volcano plot: Genus level. The red dots refer to the significant genera.

7.3 Nickel and iron addition to improve the biogas production in a two-stage system

The effect of TM on acetogenesis and methanogenesis has been widely studied. However, less research has been done about the TM effect in a two-stage system (Voelklein *et al*., 2017), especially CH_4-rich biogas production from acidogenic effluents enriched with TM. In this sense, this work is intended to bridge that knowledge gap by evaluating the effect of Fe^{2+} and Ni^{2+} addition on H_2-rich biogas production from organic solid waste and the CH_4-rich biogas production from the AE enriched with TM. The composition of microbial communities was also examined to explore the impact of TM on microbial diversity. Promotion of microbial activity by TM without reducing diversity would be valuable to reinforce bioprocess stability.

7.3.1 Biohydrogen Potential test

TM had an influence on HY and VS removal. HY in the control (without TM) was 122±2 mL H_2/gVS_{added}. The HY decreased by 89% and 88% when Ni^{2+} was added as unique TM (0.25 and 0.5 mg/L), reaching 13±1 and 14.5±0.5 mL H_2/gVS_{added}, respectively. In the study conducted by Wang and Wan, (2008) who tested Ni^{2+} concentrations from 0.01 to 50 mg/L in batch reactors, they detected a low HY of 120 mL H_2/g glucose by applying the higher Ni^{2+} concentration. In the present study, a toxic effect was detected as a consequence of Ni^{2+} supplementation due to the low HY. The addition of Fe^{2+} as sole TM displayed a less deleterious effect than Ni^{2+} on HY. The concentration of 667 mg/L Fe^{2+} led to an HY of 146±2.5 mL H_2/gVS_{added}, which is 19.67% higher than the control (Fig. 21A). In the research performed by Zhang *et al*., (2017) the HY from glucose increased by 37% regarding the control when 200 mg/L of Fe^{2+} were applied. Nevertheless, a complex substrate (OFMSW) was used for this research, occasioning differences regarding the effect of TM on HY, since the complex aqueous chemistry of the systems influences the availability of TM through precipitation and the presence of chelating agents (Demirel and Scherer, 2011).

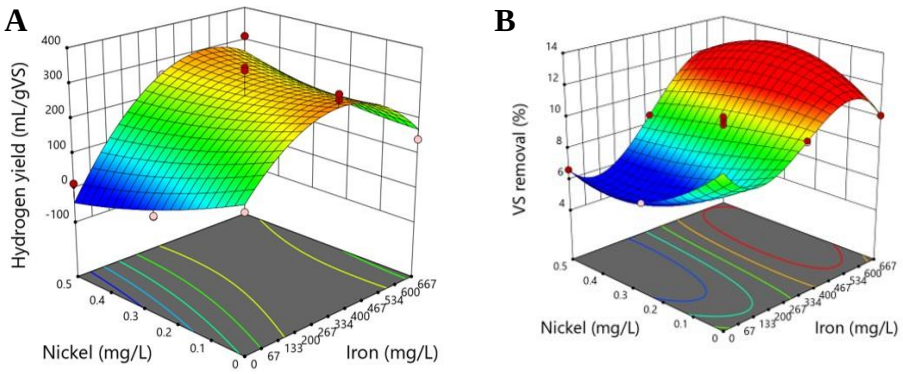


Fig. 21. A) Ni^{2+} and Fe^{2+} effect on hydrogen yield and, B) Ni^{2+} and Fe^{2+} effect on volatile solids removal.

In this study, an HY of 342 mL H_2/gVS_{added} was obtained by applying 334 mg/L of Fe^{2+} and 0.25 mg/L of Ni^{2+} corresponding to a threefold increase compared with the control. These conditions also promoted the highest concentrations of total metabolites (6.2±1.5 g/L of acetate, propionate, and butyrate). In complex substrates such as OFMSW, where the removal of VS is a crucial issue, the supplementation of TM should be considered. The highest VS removal (10.6±0.5%) was achieved with the highest Fe^{2+} concentration (667 mg/L). For comparison, in the reactors with the highest HY, the VS removal was 9.9±0.1% (Fig. 21B). In agreement with these results, in the study carried out by Chen *et al*., (2021), the addition of 5 mg/L of Ni^{2+} to H_2-producing reactors using slurry as substrate, showed a soluble COD removal of 27.69%, which was higher than the control; thus, the efficiency of substrate utilization was related to soluble COD degradation. According to Choong *et al*., (2016), besides the biogas enhancement, TM can increase the substrates' degradation efficiency, plus COD and solids removal.

7.3.2 Acidogenic reactor (first stage)

The reactor was operated for 25 cycles to allow stabilization before TM additions. Productivities of 1823±160 mL H_2/L·d and HY of 28±2 mL H_2/gVS_{added} were obtained during that period (Fig. 22). The lower HY compared with the batch tests can be partly explained by the shortest reaction time of the reactor (8 hours vs. 30 hours in batch tests). TS, VS, and COD removals were 26.5±0.8%, 15.4±0.25%, and 40±1.6% during the first 25 cycles, respectively.

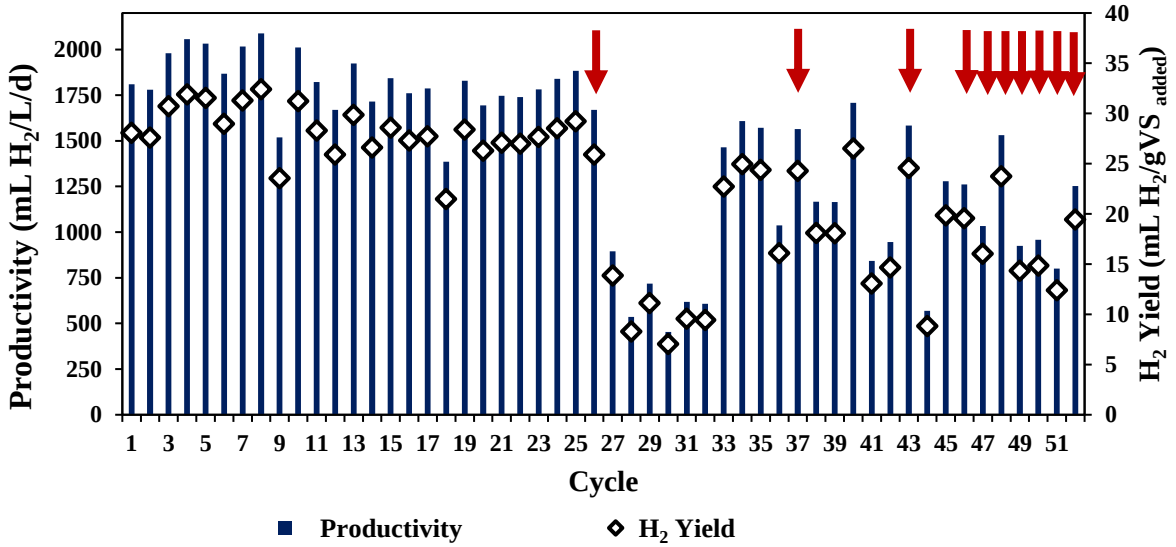


Fig. 22. Hydrogen yields and productivities obtained in the acidogenic reactor (first stage). The red arrows represent the cycles where Fe^{2+} and Ni^{2+} were added.

TM were added at cycle 26. The productivity and HY decreased at the end of the cycle. Both parameters remained low from cycles 27 to 32, achieving a productivity of 638±140 mL H_2/L·d and an HY of 10±2.2 mL H_2/gVS_{added}. In cycle 33, the productivity and HY increased. This behavior could be explained due to the acclimatization time of the microorganisms to different TM concentrations. According to Chen *et al*., (2008) and Ezebuiro *et al*., (2018), after prolonged periods, microorganisms increase their tolerance to different TM concentrations; therefore, biogas production could increase. In cycle 37, TM were supplemented. The HY was variable in a range of 8 to 25 mL H_2/gVS_{added}. TM addition (indicated by red arrows in Fig. 22) caused a transient reduction of H_2-rich biogas production differing from the results obtained in the BPH. However, in batch tests, each reactor was inoculated independently, while the acidogenic reactor was inoculated just at the beginning of the operation. Microbial communities change over time which can conduct to different results in biogas production.

In agreement with these results, García-Depraect *et al*., (2019) used a mesophilic lab-scale fermenter to produce H_2-rich biogas from tequila vinasses; the addition of $FeSO_4 \cdot 7H_2O$ did not improve the biogas production, which was related with possible changes in metabolic pathways. Low hydrogen production may be explained due to the formation of products such as lactate or butyrate, involved in the oxidation of NADH. The final hydrogen yield depends on the main metabolite pathway orientation (Cabrol *et al*., 2017; Cai *et al*., 2011). Regarding the VS removal, it increased up to 70% (Table 8) after the TM supplementation. This is an important finding since AE could be used for CH_4 production, minimizing the need to add water to dilute and reduce the OLR. Regarding the above, Ezebuiro and Körner, (2017) investigated the catalytic potentials of TM in simple and complex substrates; their results showed that TM supplementation enhances substrate hydrolysis and acidification rate and prevent inhibition due to acid accumulation.

Table 8. Results obtained in the first stage (acidogenic reactor).

Parameter	Units	Cycle (1-25)	Cycle (26-36)	Cycle (37-46)	Cycle (47-52)
pH	-	5.5±0.2	5.5±0.2	5.5±0.2	5.5±0.2
Total solids	g/L	26.5±0.8	20.3±4.4	24.4±4.9	26.6±2.2
Volatile solids	g/L	15.4±0.25	12.2±1.7	13.8±2.3	11.2±2.8
COD	g/L	34.1	42.2	36.5	39.1
Acetate	g/L	1.29±0.13	2.01±0.20	2.29±0.40	2.91±0.90
Butyrate	g/L	2.92±0.13	3.74±2.25	1.88±1.56	4.68±0.51
Total VFA	g/L	4.21±0.18	5.75±2.44	4.16±1.39	7.59±1.16
HY	mL H_2/g VS_{added}	28±2	16±7	19±5	17±4
H_2 Productivity	mL H_2/L/d	1823±160	1016±454	1209±338	1083±243

7.3.3 Methanogenic digester (second stage)

The methanogenic reactor was operated for 38 cycles. The reactor was fed with the AE without TM from cycles 1 to 12. The first OLR evaluated was 2.7 gVS/L·d since no dilutions were performed. The productivities and yields obtained in the first five cycles correspond to 1260±166 mL CH_4/L·d and 233±32 mL CH_4/gVS_{added}, respectively (Fig. 23). However, the high OLR and the short HRT impacted the performance of the process from cycle 6, quantifying low biogas production. For this reason, it was necessary to re-inoculate the reactor to pursue the operation (red line, Fig. 23). The reinoculation was carried out by adding 400 mL of anaerobic granular sludge. Before continuing with the evaluation, the reactor was operated by six cycles with an OLR of 1.6 gVS/L·d to avoid inhibition since, in anaerobic digesters, low stability is expected when a high OLR is applied (Mata-Alvarez *et al*., 1992; Shen *et al*., 2013).

The first AE enriched with TM was added from cycle 13. The specific methane yield (SMY) increased suddenly to 331±67 mL CH_4/gVS_{added}. The OLR in cycles 13 to 16 was 1.5 gVS/L·d. The process operated stably since the reactor was fed with the AE enriched with TM. In this period, the productivity was 946±77 mL CH_4/L·d. The productivities and SMY in the following cycles were stable even when the reactor operated at high OLRs of 2.4 gVS/L·d (from cycles 17-23) and 2.8 gVS/L·d (from cycles 24-30). These results support the benefit of TM addition. The optimal dose of TM varies with feedstock, and TM requirements increase with organic dry matter supply to the reactor (Pobeheim *et al*., 2011). In this sense, Wall *et al*., (2014) operated a reactor using grass silage as a substrate using high OLRs, they supplied the reactor with a mix of Co, Ni, and Fe to maintain a stable AD process. Thus, the SMY increased by 12% up to 404 mL CH_4/gVS, and the VFA removal rates also increased. In the same way, Gustavsson *et al*., (2011), investigate the effect of TM addition on lab-scale biogas tank reactors using wheat stillage as substrate at a high OLR of 4 gVS/L·d; to maintain the process stability they applied a daily supplementation of Co (0.5 mg/L), Ni (0.2 mg/L) and Fe (0.5 mg/L). The $SMY_{(OFMSW)}$ calculated from the VS concentration of the OFMSW fed in the first stage is lower than the $SMY_{(AE)}$. The reason for this difference is that the $SMY_{(AE)}$ calculated from the VS in the AE contains a part of the organic matter hydrolyzed as VFA that cannot be determined as VS. After cycle 30, the initial conditions were repeated by feeding the AE without TM at an OLR of 1.6 gVS/L·d. The productivities obtained in this period were stable in the same range as the previous condition using AE enriched with TM (1011-1363 mL CH_4/L·d). Nevertheless, the SMY increased to 442 mL CH_4/gVS_{added} in cycle 33. The results obtained in this research agree with the findings obtained by Voelklein *et al*., (2017). They compared the effect of the TM in one and two stages to determine the impact and the response of the process after the TM addition; the results obtained showed that Co, Fe, Mo, Ni, and Se restored a stable process and allowed increased loading rates. Also, the hydrolytic pre-treatment improved the SMY compared with the single stage but did not show any better resilience to nutrient deficiency.

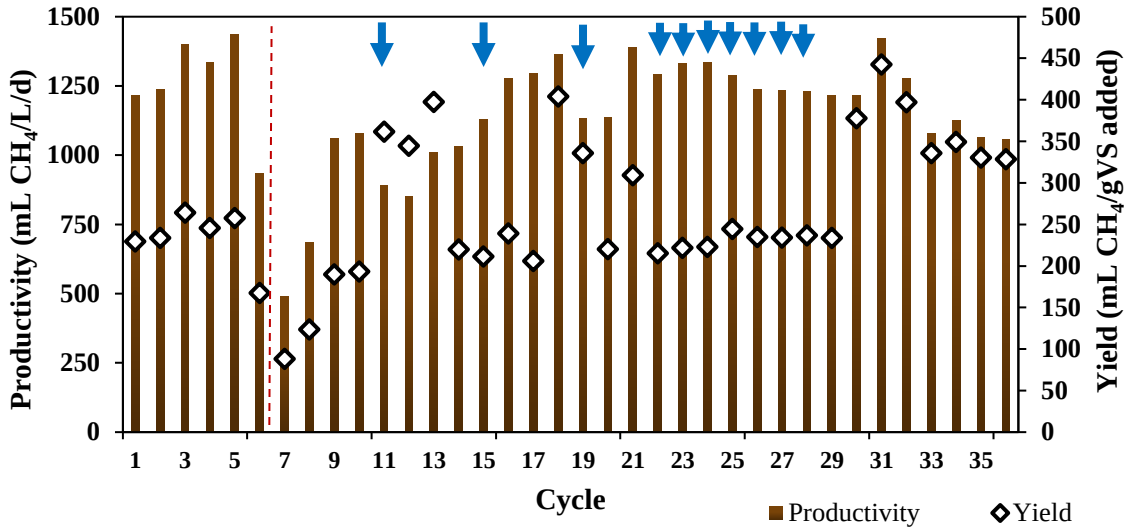


Fig. 23. Methane yields and productivities obtained in the second stage. The blue arrows represent the cycles where the acidogenic effluents enriched with TM were added.

In this last operation period, besides the SMY, the removal of TS, VS, and COD were also improved (Table 9). The digestate obtained could be used for agricultural applications (Peng *et al*., 2020). A similar effect of high removal efficiencies was reported by Ignace *et al*., (2016) who used iron powder to increase the SMY from sewage sludge; in the presence of this additive, the COD was reduced (51%-70.6%). Regarding the VFA, it is known that a possible cause of AD failure is VFA accumulation (Li *et al*., 2012). The metabolites produced at the end of the first stage were completely removed by feeding the methanogenic reactor with the AE enriched with TM. This effect was also reported by Espinosa *et al*., (1995) who achieved a reduction of propionic acid from 5,291 mg/L to 251 mg/L and an acetic acid reduction from 1,100 mg/L to 158 mg/L in a UASB reactor using a mix of Fe, Co, Mo, and Ni. In the same way, Osuna *et al*., (2003) induced the propionate degradation in an Up-flow anaerobic sludge bed reactor by the addition of a TM solution containing Fe, B, Zn, Mn, Cu, Ni, and Se. Wall *et al*., (2014) obtained low concentrations of propionic acid in a CSTR by adding Co, Fe, and Ni, under various operating conditions and with different substrates. Similar VFA removals were obtained by applying AE enriched with TM even though the concentrations and operational conditions differed. After the TM addition, it was possible to achieve process stability, obtaining high yields and productivity even when operating at high OLR. These results were also achieved due to the physical separation of the reactors since the environmental conditions of the groups of microorganisms that intervene in each stage may differ widely.

Table 9. Results obtained in the second stage in the methanogenic reactor.

Parameter	**Units**	**Cycle (1-6)**	**Cycle (13-16)**	**Cycle (17-23)**	**Cycle (24-30)**	**Cycle (31-38)**
Ni	mg/gTS	0	2.372	3.738	4.224	5.044
Fe	mg/gTS	0	8.246	10.754	11.538	12.288
OLR	gVS/L/d	2.7	1.5	2.4	2.8	1.6
pH	-	7.9±0.07	8.06±0.03	8.1±0.03	8.1±0.08	8.2±0.04
TS	g/L	17.0±3.0	14.8±1.0	15.8±3.6	11.5±3.5	9.4±1.1
VS	g/L	5.9±1	8.3±3.2	9.6±1.2	5.7±1.4	4.4±1.8
COD	g/L	6.2	11.5	10.8	11.2	8.6
Total VFA	mg/L	Not detected	Not detected	Not detected	Not detected	Not detected
$SMY_{(AE)}$	mL CH_4/gVS_{added}	233±32	331±67	275±70	230±10	350±57
Productivity	mL CH_4/L/d	1260±166	946±77	1246±105	1278±42	1181±119

7.3.4 Microbial community analysis

The acidogenic reactor was inoculated with thermally pretreated sludge, leading to a predominance of ASV affiliated with *Megasphaera* belonging to the phyla Firmicutes. *Megasphaera* spp. are non-spore forming obligate anaerobe encoding [FeFe]-hydrogenase to produce H_2 (Cabrol *et al*., 2017; Søndergaard *et al*., 2016; Wang *et al*., 2018). A low relative abundance of the phylum Proteobacteria (7%) and Bacteroidota (3%) were also observed. ASV encompassing both phyla were not detected between cycles 26 to 46 after TM additions. Other compositional changes in microbial communities were observed in Firmicutes. A succession from ASV affiliated with *Megasphaera* spp. after inoculation to ASV affiliated with *Succiniclasticum* spp. (39%) was noticed in the last operation cycles. This genus has been previously reported in changes in hydrogen metabolism to propionate production from succinate (Hahnke *et al*., 2016; Lopes *et al*., 2016). Taken together, the results indicate a potential influence of Fe^{2+} and Ni^{2+} on microbial communities in addition to exerting an effect on VS removal and VFA in AE supplied to the second stage of AD.

Contrasting microbial community structures were observed among acidogenic and methanogenic reactors (Fig. 24). Distribution profile of ASV affiliated to *Megasphaera* spp. in the first stage and, *Proteiniphilum* spp., *Thermovirga* spp., *DMER64* spp., *Anaerovorax* spp., and Syntrophomonas spp., in the second stage, contributed to contrasting microbial communities, with higher relative abundance observed in the first and second stage, respectively (Fig. 25). *Proteiniphilum* spp. is facultative anaerobic bacteria presumably generating acetic and propionic acids as main fermentation products (Hahnke *et al*., 2016). *Thermovirga* spp. has been previously identified as a sulfate/Fe (III)- respiring gene with the ability to proceed with acetate oxidation (Wang *et al*., 2023). The archaeal abundance in the methanogenic effluent was negligible due to the sampling strategy applied. The samples were taken after the sedimentation of the SBR; therefore, the archaeal populations inside the sedimented granular sludge were not detected.

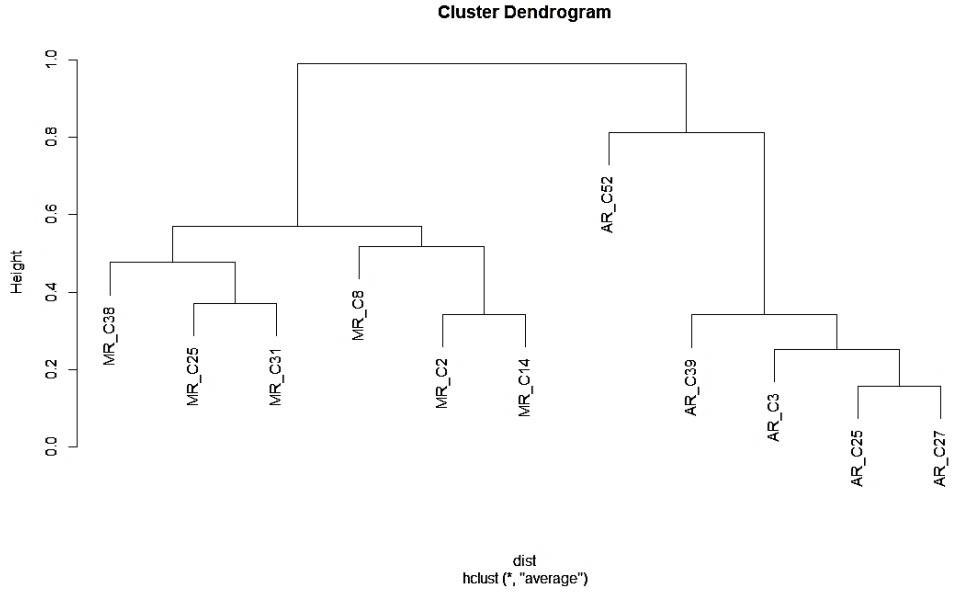


Fig. 24. Cluster dendrogram Based on Bray-Curtis distance for SBR in the first and second stages. (AR: Acidogenic reactor or first stage, MR: methanogenic reactor or second stage, C: cycle)

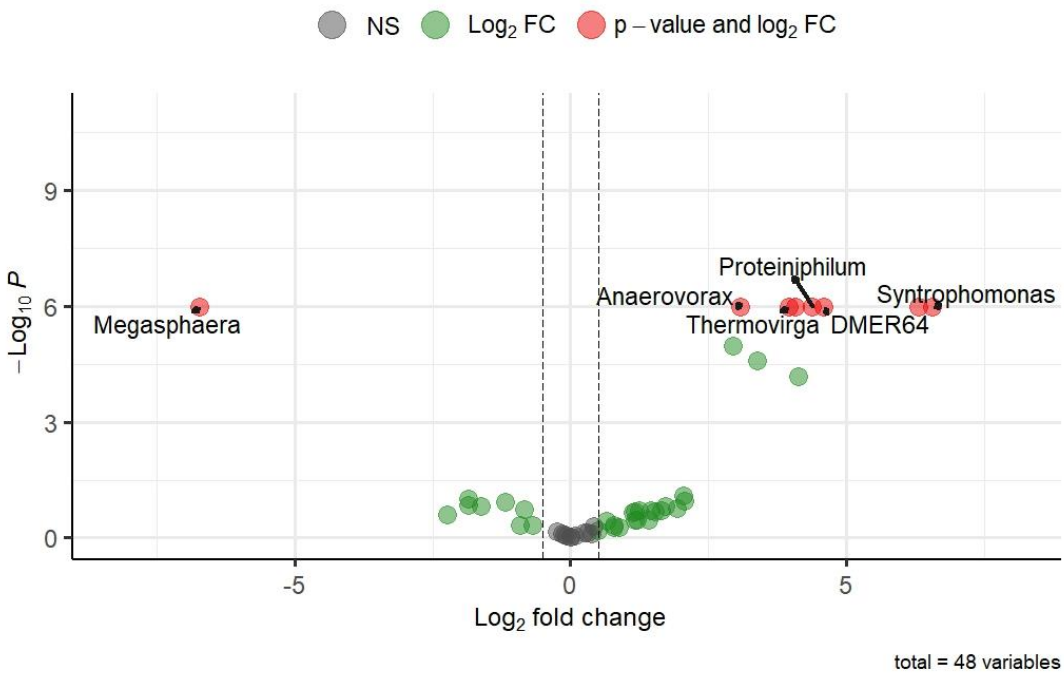


Fig. 25. Volcano plot for SBR in the first and second stages. The red dots refer to the significant genera.

7.4 Assessment of biogas production concerning trace metals

There are challenges related to biogas production from OFMSW e.g., the difficulty of starting up the process, longer stabilization, inhibition due to the generation of toxic compounds. Achieving high productivity is limited to the stable operation of the system. In the case of H_2-rich biogas production, when the HRT is longer than 2 days, pH tends to drop due to the accumulation of VFA. If operating with a shorter HRT, the process may require the addition of trace metals to obtain higher productivity and yield. In this sense, Fig. 26 presents a decision tree based on the results obtained during the H_2-rich biogas production.

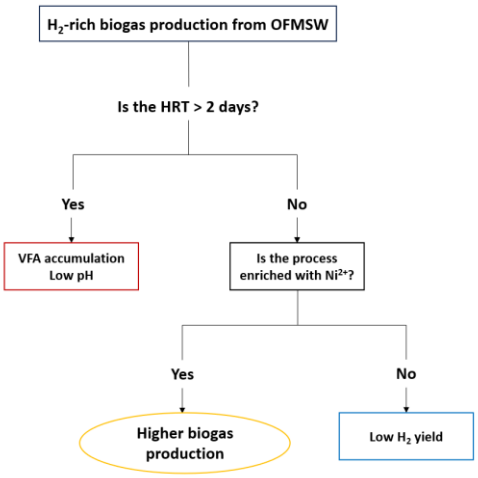


Fig. 26. Decision tree concerning trace metals supplementation for hydrogen-rich biogas production from OFMSW.

In the case of CH_4-rich biogas production, the one-stage AD process has been widely studied. However, methane-rich biogas production from acidogenic effluents is still under study. The AE may present different VS concentrations; thus, to adjust the OLR, it is necessary to add water. Nevertheless, TM can reduce water demand. Fig. 27 shows a decision tree based on the results obtained when the AE were used as a substrate. When AE are fed undiluted (as is the case in the present thesis), the process may be inhibited. The results showed that when using AE enriched with Fe^{2+} and Ni^{2+}, it is possible to operate stably considering the variations in the OLR and at a short HRT.

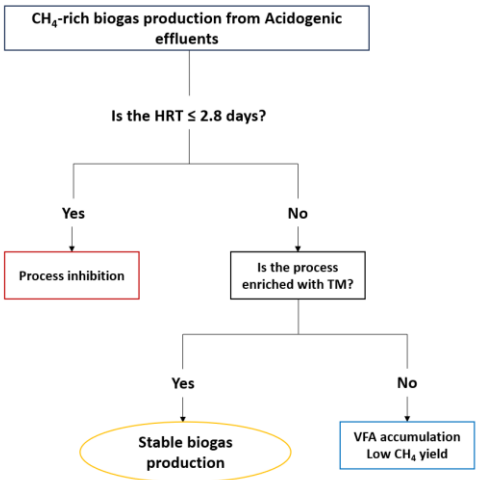


Fig. 27. Decision tree concerning trace metals supplementation for methane-rich biogas production from acidogenic effluents.

8 CONCLUSIONS

The addition of trace metals (Ni^{2+} or Ni^{2+} and Fe^{2+}) in hydrogen-producing reactors exerts an effect on the production of hydrogen-rich biogas. Under the proper operating conditions, TMs effect can be positive by increasing hydrogen yields. Trace metal-enriched acidogenic effluents improve the stability of methane-producing reactors by increasing biogas yields and avoiding process inhibition. The conclusions corresponding to each specific objective are presented below.

For the effect of nickel concentration on biohydrogen production: organic solid waste vs. glucose, the batch tests using glucose as a reference substrate showed the highest H_2 production when 2 mg Ni^{2+}/g $VS_{inoculum}$ was applied. H_2 production increased by 34.4% compared to the control without nickel. Hence, this value could be taken as a reference to stimulate H_2 production by adding nickel to substrates lacking this trace metal. When OFMSW was used as a carbon source, and different doses of nickel were applied, the final accumulated volume decreased. In the SBR operation, the effect was similar since higher biogas production and hydrogen yields were obtained without the addition of Ni^{2+}. However, it must be considered that the OFMSW contains a nickel concentration of 0.17 ± 0.06 mg Ni/g TS; therefore, it is possible that there was not a lack of this trace metal during the DF process. Adding 0.5 mg Ni^{2+}/g $VS_{inoculum}$ decreased acetate and butyrate production and increased caproate production. The addition of different concentrations of Ni^{2+} increases the concentration of Ni in the digestate; however, it could be recirculated to the system or used to produce methane since this trace metal has a vital role in anaerobic digestion in the growth of all methanogens and the synthesis of cofactor F_{430}. It is necessary to test the effect of Ni^{2+} focusing on the microbial population dynamics and its metabolic pathways related to H_2 and VFA production in long-term operated bioreactors using OFMSW as substrate.

In the two-stages system subjected to nickel addition compared with a conventional digester, the productivities and yields obtained in TSAD increase by 72% and 144%, respectively, which proves that TSAD improves the methane production besides the H_2-rich generation and increases the remotion of TS, VS and COD in the digestate. The results proved that Ni^{2+} addition improves the system's stability, allowing high biogas production. Changes in microbial communities' composition at phylum level were detected in each stage and the genus were correlated with the operational parameters. Cloacimonadia and JS1 were the most significant class in the first stage, and Methanobacteria and Actinobacteria was the predominant class in the second stage. Meanwhile, genus *LNR A2-18* was the most significant in the first stage, and *Methanobrevibacter* and *Methanosphaera* were the predominant genera in the second stage.

Finally, the nickel and iron addition improved the biogas production in a two-stage system. The optimal TM concentrations that increase the HY in BHP were obtained as 0.25 mg Ni^{2+}/L and 334 mg Fe^{2+}/L. However, when the acidogenic reactor (first stage) was supplied with the TM, the HY, and productivity decreased which was related to a high VS removal detected after each TM addition. Regarding the second stage, the use of undiluted effluents to feed the methanogenic reactor caused the fast decay of the process. Nevertheless, when the reactor was fed with the AE enriched with TM, a sudden increase in the SMY was detected, besides, it was possible to maintain the stability of the process, avoiding inhibition and obtaining high productivity. Although in the first stage, the use of TM did not enhance the H_2 productivities and HY, the use of AE enriched with TM in a two stages system could have great potential to avoid the use of water to dilute the effluents and to achieve process stability. Important changes in microbial communities were detected, especially in the methanogenic reactor. The H_2-rich biogas in the first stage was influenced by *Megasphaera* genus, meanwhile, *Proteiniphilum* spp., *Thermovirga* spp., *DMER64* spp., *Anaerovorax* spp., and *Syntrophomonas* spp. were the most significant genus in the second stage. The results indicate a potential influence of Fe^{2+} and Ni^{2+} on microbial communities in addition to exerting an effect on VS removal and VFA in AE supplied to the second stage of AD.

9 PERSPECTIVES

- This research was focused on the study of the effect of TM (Ni^{2+} and Fe^{2+}) in the DF process. TM supplementation affects not only the production of hydrogen-rich biogas but the production of metabolites such as caproate. Future research should focus on the study of changes in metabolic pathways.
- The addition of TM affects the microbial communities in the hydrogen and methane-producing reactors. Therefore, it is necessary to evaluate if these long-term changes are positive and how they correlate with operational parameters such as organic loads, pH, and matter removal organic.
- The use of TM to recover the stability of anaerobic digesters has been verified, as well as its effect on increasing the production of biogas rich in methane. However, the frequency of the addition of TM should continue under study since the necessary amount of mineral solution could be lower, as well as the accumulation of TM in the digestates.
- This thesis focused on TM addition in the dark fermentation process (first stage). However, the effects of TM are more beneficial in the second stage. Therefore, future research could test the addition of metals in acidogenic effluents for methane-rich biogas production, especially when operating at low HRT.
- It is recommended to monitor the activity of the main metalloenzymes and coenzymes both in batch and SBR operation. In this way, it could be possible to know if the addition of trace metals has a direct effect on the increase in enzymatic activity or if there are other physicochemical factors limiting biogas production.

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