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Iron and Nickel Supplementation Exerts a Significant Positive Effect on the Hydrogen and Methane Production from Organic Solid Waste in a Two-Stage Digestion

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Abstract

Two-stage anaerobic digestion and trace metals (TM) supplementation are promising techniques to improve biogas production. Fe^{2+} and Ni^{2+} can improve process stability since they are part of the cofactors of enzymes and microorganisms' growth. This work attempted 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 acidogenic effluents (AEs) enriched with TM. The TM concentrations that enhanced the hydrogen yield in the batch were 0.25 mg/L of Ni^{2+} and 334 mg/L of Fe^{2+}. These concentrations were evaluated in a two-stage system. The substrate for the batch tests and fermentative reactor (first stage) was OSW. The AE generated in the first stage was the substrate to produce CH_4-rich biogas in the second stage. In the first stage, the productivity achieved was 1823 ± 160 mL H_2/L/day. However, TM supplementation decreased productivity by 65% since the VS removal increased. *Megasphaera* genus predominated in the first stage. Regarding the methanogenic reactor, the undiluted AE without TM caused the fast decay of the process. Nevertheless, the reactor operated stably after using AE enriched with TM as a substrate, and CH_4 yields increased by 42%. The highest productivity achieved in the second stage was 1278 ± 42 mL CH_4/L/day, operating with an organic loading rate of 2.8 gVS/L/day. The genera *Proteiniphilum*, *Thermovirga*, *DMER64*, *Anaerovorax*, and *Syntrophomonas* predominated in the second stage. In conclusion, AE enriched with TM can be used to recover the stability of anaerobic digesters, increasing methane production.

Keywords Hydrogen-rich biogas · Methane-rich biogas · Organic solid waste

Introduction

Organic solid waste (OSW) as food waste can be an economical feedstock for biogas production. It contains a relatively high total solids (TS) content (~ 20%) and volatile solids (VS) (~ 90% of TS) [1]. OSW can support the production of up to 110 m^3 of biogas/t in a single stage [2]. Dark fermentation (DF) and anaerobic digestion (AD) are attractive technologies for biogas production (H_2 and CH_4). H_2 production from OSW is challenging. However, different strategies have been explored to increase yields, such as maintaining the pH in acidic conditions (5.5), operating in mesophilic temperatures, macro and micronutrient supplementation, or selecting a pretreated inoculum to prevent the proliferation of methanogenic archaea [3]. Single-stage anaerobic digestion of OSW deals with challenges such as volatile fatty acid (VFA) accumulation, insufficient buffering capacity, and ammonia inhibition. In this way, two-stage anaerobic digestion is a promising technique to improve stability and methane production at high organic loading rates (OLR) [4].

Nevertheless, a generalized application of this technique is limited by low biogas yields [5]. One of the most important strategies to enhance biogas yields is to ensure an appropriate nutritional balance for a stable bioconversion of organic substrates and recovering energy [6]. For instance, trace metal (TM) deficiency could limit the AD process since they are part of the cofactors of enzymes and

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microorganisms' growth implicated in the AD process [7]. 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 that are also required [8].

TM concentration is also necessary in DF. In this process, molecular H_2 is produced during microbial fermentation to dissipate excess reductant, primarily generating NAD^+ from NADH [9]. This process is accomplished by metalloenzymes called hydrogenases that contain nickel and iron in their active site. Hydrogenases are mainly classified into three classes according to their metal content, namely [Fe]-, [Fe–Fe]-, and [Ni–Fe]-hydrogenases [10]. [Ni–Fe]-hydrogenases are widely distributed among bacteria, and both nickel and iron bioavailability have essential effects on fermentative H_2 yields [11]. On the other hand, in the AD process, Fe^{2+} and Ni^{2+} also have an important role. Ni^{2+} is the center of coenzyme F_{430} in methyl-coenzyme M reductase, which catalyzes the methyl-CoM to CH_4 in methanogenesis [12]. Fe^{2+} is involved in redox reactions as an electron transfer carrier in the growth and metabolism of microorganisms, to accelerating the hydrogen transfer process [13]. However, TM´s beneficial or inhibitory effects depend on the TM concentrations due to metal toxicity [14]. According to Bozym et al. [15], the toxic threshold for Ni^{2+} in fermented wastes is 10 mg/L. In the case of iron, the daily supplementation of 400 mg/L could inhibit the growth and metabolism of microorganisms [16]. In this sense, identifying the dose of TM that increases yields is not the only concern, but the frequency of addition to avoiding inhibition of the system.

The effect of TM on acetogenesis and methanogenesis has been widely studied. However, more research needs to be performed about the TM effect in a two-stage system, especially CH_4-rich biogas production from acidogenic effluents enriched with TM. Since AE contains a high VFA concentration, it can be used to produce CH_4-rich biogas in a second stage. VFA concentrations of AE may present different characteristics depending on the type of substrate, operating conditions, or if TM were supplemented. For instance, Chen et al. [17] obtained AE with a concentration of 973 mg/L total VFA in the control.

Meanwhile, the supplementation of 5 mg/L Ni^{2+} decreased the VFA concentration to 880 mg/L total VFA. On the other hand, Zhang et al. [18] carried out batch tests, obtaining different compositions in the acidogenic effluents. The control contained 198 mg/L, 1087 mg/L, 144 mg/L, 2186 mg/L ethanol, acetate, propionate, and butyrate, respectively. The AE enriched with 300 mg/L Fe contained 135 mg/L, 1112 mg/L, 129 mg/L, and 2121 mg/L for ethanol, acetate, propionate, and butyrate, respectively. 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 in the first stage and the CH_4-rich biogas production from the AE enriched with TM in the second stage. The composition of microbial communities was also examined to explore the impact of TM on microbial diversity. Promoting of microbial activity by TM without reducing diversity would be valuable to reinforce bioprocess stability.

Methodology

Substrate and Inoculum

The substrate used in this study was OSW. It was obtained from the regional municipal market of Queretaro, Mexico. The OSW was composed of green vegetables including lettuce, cabbage, chard, and prickly pear (64.6%), tomato (7.1%), potato (6.3%), carrot (5.4%), cauliflower (5.2%), watermelon (3.1%), chicken (2%), broccoli (1.6%), onion (1.4%), sweet potato (1.3%), papaya (1%), and melon (0.9%) on a wet weight basis. The OSW was shredded in an industrial blender, stored in plastic bags, and frozen at − 4 °C until use. The concentration of total solids (TS) and volatile solids (VS) were 8.8 ± 1.28% and 7.5 ± 1.7%, respectively, and the chemical oxygen demand (COD) was 56.7 ± 0.14 g/L. Fe and Ni concentration were 8.7 ± 0.05 mg Fe/gTS and 0.2 ± 0.04 mg Ni/gTS. Anaerobic granular sludge collected from a full-scale up-flow anaerobic sludge blanket was used as inoculum of the methanogenic reactor. The sludge had a composition of 183 ± 28 gTS/kg and 171 ± 25 gVS/kg. Fe and Ni concentrations were 0.82 ± 0.02 mg Fe/gTS and 0.0018 ± 0.04 mg Ni/gTS. For the inoculation of the acidogenic reactor and batch test to produce H_2-rich biogas, the sludge was thermally pretreated at 105 °C for 24 h to eliminate H_2-consuming methanogens [19] and select spore-forming bacteria, including *Clostridium* genus considered the more efficient H_2 producers [20]. The composition of the pretreated granular sludge was 980 gTS/kg and 780 gVS/kg.

Batch Experimental Setup

Batch tests were carried out to measure the biohydrogen potential (BHP) according to the protocol established by Carrillo-Reyes et al. [21] 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 used was OSW. The substrate/inoculum rate (S/I) was 2.7 in terms of VS, and the substrate concentration was 15 gVS/L. To evaluate the effect of TM, the concentrations were proposed based on a literature review where Fe^{2+} and Ni^{2+} were used to increase the H_2-rich biogas production [11, 22, 23]. Fe^{2+} and Ni^{2+} were added as $FeSO_4 \cdot 7H_2O$ and $NiCl_2 \cdot 6H_2O$, respectively.

Experimental Design and Data Analysis

The experiment was carried out for 30 h. The effect of experimental variables (Fe^{2+} and Ni^{2+}) on hydrogen yield (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. These Ni^{2+} concentrations expressed as mgNi^{2+}/gTS are 0, 0.0045, 0.0097, 0.014, 0.019, 0.024, and 0.028. In the case of Fe^{2+}, the concentrations analyzed were 0, 111, 222, 334, 445, 556, and 667 mg/L. These concentrations expressed as mg Fe^{2+}/g TS are equivalent to 0, 6.3, 12.6, 18.9, 25.3, 31.6, and 37.9. The data were analyzed with an ANOVA. The response variables were the hydrogen yield, and VS. Samples were run in replicates to allow statistical tests on the data. The H_2 cumulative volume was adjusted to the modified Gompertz equation shown in Eq. (1), where H_{max} (in mL) represents the H_2 volume, the maximum flowrate is represented by R_{max} (in mL/min), the lag period λ (in min). The results were analyzed by using MATLAB R2021b. The parameter T90 was estimated according to the time in which 90% of H_2 was produced.

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

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 was 5 min, while the settling and settling time was 50 min. The reactor operated with an OLR of 60 gVS/$L_{reactor}$/day 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 was added according to the results obtained in the batch tests. Both TMs were added simultaneously to study the interaction of metals with biogas production, organic matter removal, and microbial communities. The doses applied corresponded to 0.25 mg/L of Ni^{2+} and 334 mg/L of Fe^{2+} since those concentrations enhanced the H_2 production in the batch test. The TM supplementation was carried out in cycles 26, 37, 43, and 46–52.

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 min, 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, an initial pH of 7.5 ± 0.2, a temperature of 37 ± 0.5 °C, and an HRT of 2.8 days. 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.

Analytical Methods

COD determination was performed using the Hach 435 method. TS and VS were measured in triplicate according to standard methods (2540G) [24]. VFA was quantified by gas chromatography with a flame ionization detector (FID, Agilent Technologies 7890B) as reported by Cardeña et al. [25]. Biogas composition was determined by gas chromatography using an equipped SRI 8610C, SRI instrumental, USA) using 5 mL of sample, in accordance with Buitrón et al. [26]. Flame absorption spectrometry quantifies nickel and iron concentrations in the sludge, OSW, AE, and digestates according to standard methods (3111A).

Microbial Community Analysis

Samples were taken to analyze planktonic microbial communities from the acidogenic effluents at the operation cycles 3, 25, 27, 39, and 52, with the first two samples corresponding to background conditions without TM addition. The samples from the methanogenic reactor were taken from cycles 2, 8, 14, 25, 31, and 38; the first three samples were taken from the digestates without TM addition. All the samples were stored at -20 °C. DNA was extracted using the DNeasy Power Soil Kit (Qiagen, Germany). The V4 region of bacterial and archaeal 16S rRNA gene was PCR-amplified with primers 515F (5′-TCGTCGGCAGCGTCAGATGTG TATAAGAGACAGTGYCAGCCGCCGCGGTAAHAC-CVGC-3′) and 806R (5′-GTCTCGTGGGCTCGGAGATGT GTATAAGAGACAGGGACTACVSGGGTATCTAAT-3′) with Illumina adapter. 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 10 × 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 [27] 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™ PicoGreen® dsDNA Assay (Invitrogen-P7589). Finally, the pool of sequencing libraries was shipped to the Centre d'Expertise et de Services Génome Québec (Montreal, Quebec, Canada) for sequencing with the Illumina MiSeq PE-250 platform.

Raw sequence reads (57,434) were processed in R (v. 4.2.1). Primer sequences were removed with Cutadapt (v.2.1) before subsequent quality control was performed with the package DADA2 package (v. 1.24.0). Default parameters were utilized in the pipeline, except for the trimming of forward and reverse sequences set at 230 bp and 230 bp, respectively. Taxonomy was assigned to amplicon sequence variants (ASVs) based on the Silva database (v. 138.1). A total of 37,971 reads clustered into 231 ASV were obtained before applying two filters. ASV for which the total abundance in all samples was less than 0.005% of total reads and ASV detected in less than 83.3% of samples were removed before downstream analyses. Beta diversity was examined with the package phyloseq (v. 1.36. 0). The potential impact of the bioreactor stage and TM addition on beta diversity was examined by a cluster analysis representing pairwise Bray–Curtis dissimilarity among samples. The package ANCOM-BC (v. 1.6.4) was utilized to identify responsive taxa. An analysis of compositions of microbiomes with bias correction (ANCOM-BC-2) was performed to identify genera displaying different relative abundance among samples collected in the first-stage and the second-stage reactors. The output of the analysis was visualized in a volcano plot. The package ggplot2 (v. 3.1.3) was used to generate the plots. Raw reads were deposited to the Sequence Read Archive repository of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/bioproject/910650) in the BioProject PRJNA910650.

Results and Discussions

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. The ANOVA showed that Fe^{2+} and Ni^{2+} significant effected the HY ($p < 0.05$, Table S1, Supplementary material). In the study conducted by Wang et al. [23], 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. 1A). In the research performed by Zhang et al. [18], the HY from glucose increased by 37% regarding the control when 200 mg/L of Fe^{2+} were applied. Nevertheless, a complex substrate (OSW) 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 [14].

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 OSW, where the removal of VS is a crucial issue, the supplementation of TM should be considered. TM significantly affected the

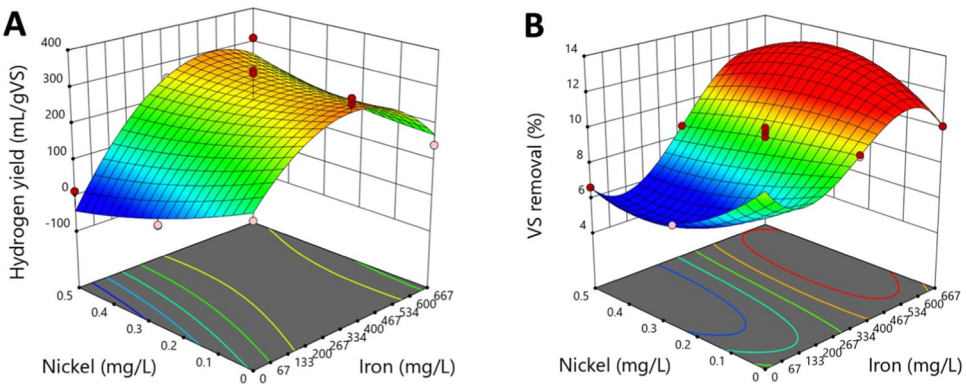

Fig. 1 **A** Ni^{2+} and Fe^{2+} effect on hydrogen yield. **B** Ni^{2+} and Fe^{2+} effect on volatile solids removal

VS removal ($p < 0.05$, Table S2, Supplementary material). 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%; this behavior is shown in Fig. 1B. In agreement with these results, in the study carried out by Chen et al. [17], 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. [28], TM can increase the substrates' degradation efficiency besides the biogas enhancement, plus COD and solids removal.

The effect of TM on the kinetic parameters of H_2 production was analyzed by fitting the data to the modified Gompertz equation (Supplementary material 1). Table S3 shows the results of the parameters H_{max}, R_{max}, and the phase lag (λ). Concerning the maximum H_2 production and maximum production rate, the model showed an increase in both cases when applying the highest concentrations of Fe^{2+} (< 334 mg/L), even with the lowest concentrations of Ni^{2+}. The longer lag phases correspond to the reactors with the highest H_2 production. In contrast, the lower lag phases correspond to the reactors where Ni^{2+} was applied as the only TM. However, the H_2 production was the lowest in these reactors. The time required to produce 90% of H_2 (T_{90} parameter) is shown in Table S3. T_{90} was ≥ 33 h in the reactors with the highest H_2 production. Conversely, T_{90} is ≤ 23 h in the reactors with the lowest H_2 production. TM influenced the T_{90} since the difference between the reactors with a high H_2 production concerning the control was 13 h.

Acidogenic Reactor (First Stage)

The reactor was operated for 25 cycles to allow stabilization before TM additions. As is shown in Fig. 2, the productivities and yields obtained in that period correspond to 1823 ± 160 mL $H_2/L_{reactor}$/day and HY of 28 ± 2 mL H_2/gVS$_{added}$, respectively. The lower HY compared with the batch tests can be partly explained by the shortest reaction time of the reactor (8 h vs. 30 h 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.

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 $H_2/L_{reactor}$/day and an HY of 10 ± 2.2 mL H_2/gVS$_{added}$. In cycle 33, productivity and HY increased. This behavior could be explained due to the acclimatization time of the microorganisms to different TM concentrations. According to Cheng et al. [5] and Ezebuiro et al. [29], 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. 2) 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. It is important to highlight the system's resilience since after each decrease in productivity and yields, the system tended to recover, thus avoiding inhibition. Microbial communities change and adapt over time, which can result in different results in biogas production.

In agreement with these results, García-Depraect et al. [30] 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 H_2 production may be explained by the formation of products, such as lactate or butyrate, involved in the oxidation of NADH. The final H_2 yield depends on the main metabolite pathway orientation [31, 32]. Regarding the VS removal, it increased up to 70% (Table 1) 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 [33] investigated the catalytic potentials of TM in simple and complex substrates; their results showed that

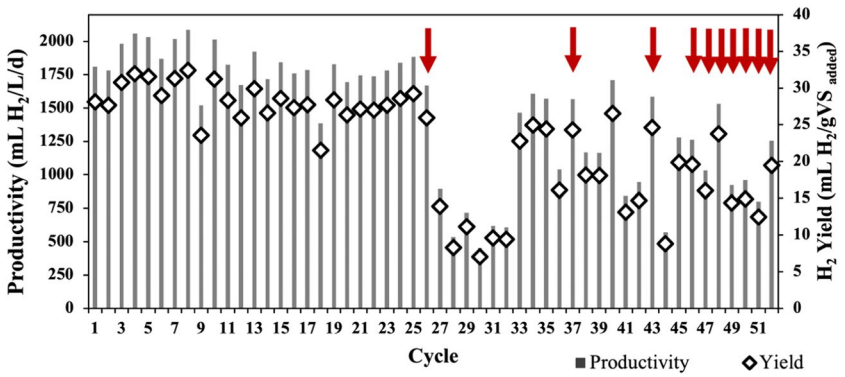

Fig. 2 Hydrogen yields and productivities obtained in the acidogenic reactor (first stage). The red arrows represent the cycles where TM were added. (334 mg/L Fe^{2+} and 0.25 mg/L $Ni.^{2+}$)

Table 1 Results obtained in the in the fermentative 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
YH_2	mL H_2/g VS_{added}	28 ± 2	16 ± 7	19 ± 5	17 ± 4
H_2 productivity	mL H_2/L/day	1823 ± 160	1016 ± 454	1209 ± 338	1083 ± 243
Ni	mg/g TS	2.37	3.74	4.22	5.04
Fe	mg/g TS	8.24	10.75	11.54	12.29

TM supplementation enhances substrate hydrolysis and acidification rate and prevents inhibition due to acid accumulation. The acetate and butyrate showed to be the principal metabolites generated (Table 1). However, metabolites such as propionate, valerate, ethanol, etc. were detected in concentrations lower than 20 mg/L. The metabolite production can be associated with microbial diversity (e.g., related to a low of microbial diversity in our test, showing few dominant species in the process) and operation parameters promoting H_2 generation.

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/$L_{reactor}$/day since no dilutions were performed. The productivities and yields obtained in the first five cycles correspond to 1260 ± 166 mL CH_4/$L_{reactor}$/day and 233 ± 32 mL CH_4/gVS_{added}, respectively, as is shown in Fig. 3. 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 in Fig. 3). 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/$L_{reactor}$/day to avoid inhibition since, in anaerobic digesters, low stability is expected when a high OLR is applied [34, 35]. This period of recovery was not considered for the statistical analysis. The evaluation continued in cycle 7.

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/day. 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/day. The productivities and SMY in the following cycles were stable even when the reactor operated at high OLRs of 2.4 gVS/$L_{reactor}$/day (from cycles 17 to 23) and 2.8 gVS/$L_{reactor}$/day (from cycles 24 to 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 [36]. In this sense, Wall et al. [37] operated a reactor using grass silage as a substrate using high OLR, supplying 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. [38] investigate the effect of TM

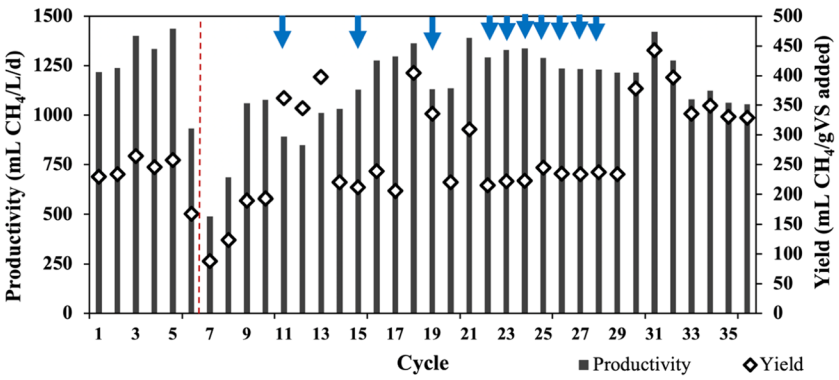

Fig. 3 Methane yields and productivities obtained in the methanogenic reactor (second stage). The blue arrows represent the cycles where Fe^{2+} and Ni^{2+} were added. The red dotted line represents the reinoculation

addition on lab-scale biogas tank reactors using wheat stillage as substrate at a high OLR of 4 gVS/L/day; 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 results were similar even when the TM concentrations differed from those evaluated in this project. Even when the TM concentrations differed from those evaluated in this project. It is possible that reactors do not require daily TM supplementation since it depends on OLR, type of operation, and substrate.

After cycle 30, the initial conditions were repeated by feeding the AE without TM at an OLR of 1.6 gVS/L/day. 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/day). Nevertheless, the SMY increased to 442 mL CH_4/gVS_{added} in cycle 33. In this way, we can argue that TM can be used to maintain the stability of an anaerobic digester. Methane production from TM-enriched AE was favorable compared to AE without TM supplementation. In a similar study conducted by Voelklein et al. [39], the effect of the TM was contrasted in one and two stages to determine the impact and the response of the process after the TM addition. The results showed that TM restored a stable operation and allowed increased loading rates. Even though the type of experiment is not the same as the one carried out in the present research, the results regarding the benefits of TM were similar.

The effect of trace metals on biological activity is highly dependent on the bioavailable fractions of metals rather than the total amount. According to Vintiloiu et al. [40], the chemical speciation of metals changes during complex chemical or biochemical reactions such as the precipitation with S^{2-}, CO_3^{2-}, and PO_4^{3-} and the formation of inorganic and organic complexes, which might obstruct the uptake of essential trace elements by methanogens. The effect of chelating agents has been studied to overcome the challenge of trace metal precipitation during methanogenesis. In the research carried out by Zhang et al. [41], biogas production from food waste improved by EDDS addition. This chelating agent uptakes metals' bioavailability for microbial communities and stimulates the growth and metabolism of methanogens. In this sense, future research should explore the possibility of increasing TM bioavailability by adding chelating agents.

In the last period (cycles 31 to 38), besides the SMY, the removal of TS, VS, and COD was also improved (Table 2). The digestate obtained could be used for agricultural applications [42]. A similar effect of high removal efficiencies was reported by Ignace et al. [43], 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 [44]. 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. [45], who achieved a reduction of propionic acid from 5291 to 251 mg/L and an acetic acid reduction from 1100 to 158 mg/L in a UASB reactor using a mix of Fe, Co, Mo, and Ni.

In the same way, Osuna et al. [46] induced the propionate degradation in an up-flow anaerobic sludge bed reactor by adding a TM solution containing Fe, B, Zn, Mn, Cu, Ni, and Se. Wall et al. [37] 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. Some microorganisms have flexible metabolisms, as in the case of clostridial species, which, depending on the operating conditions or TM supplementation, can change their metabolism from the production of H_2, acetate, and butyrate to the production of solvents such as ethanol [47]. Similarly, in methanogenesis, the presence of TM raises the growth of methanogenic microorganisms and the synthesis of metalloenzymes necessary

Table 2 Operational data obtained in the in the methanogenic reactor

Parameter	Units	Cycle (1–6)	Cycle (13–16)	Cycle (17–23)	Cycle (24–30)	Cycle (31–38)
Ni	mg/g TS	0	2.37	3.73	4.22	5.04
Fe	mg/g TS	0	8.25	10.75	11.54	12.29
OLR	g VS/L/day	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	mL CH_4/g VS_{added}	233 ± 32	331 ± 67	275 ± 70	230 ± 10	350 ± 57
Productivity	mL CH_4/L/day	1260 ± 166	946 ± 77	1246 ± 105	1278 ± 42	1181 ± 119

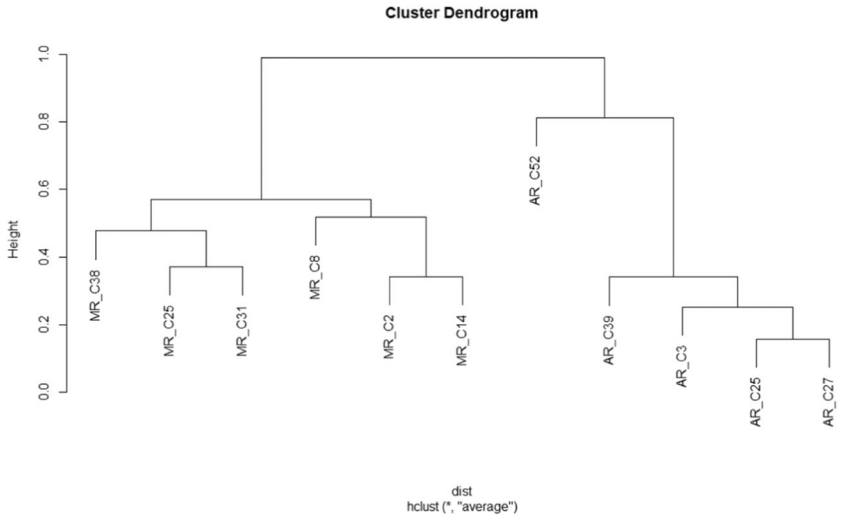

Fig. 4 Cluster dendrogram for SBR in the first and second stages (MR, methanogenic reactor; AR, acidogenic reactor, the numbers indicate the operating cycles)

for methane production. In this way, the consumption of VFA is enhanced. 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.

The net energy gain was estimated with an energy balance, according to Jimenez-Ocampo [2]. This balance did not consider the energy loss due to pumping or heat dissipation due to the short operating time of the pumps and the reactor's jacket. The energy generated by the two-stage system (H_2-rich biogas plus CH_4-rich biogas) without TM supplementation was 70.01 ± 9.8 kJ/day. Nevertheless, the inhibition of the methanogenic reactor after cycle 5 caused a cero biogas production. Therefore, the energy gain of H_2-rich biogas corresponds to 19.7 ± 1.7 kJ/day. The energy gain when TM were added to both reactors corresponds to 100.7 ± 12.2 kJ/day. Considering these values, the increase in the net energy gain with TM supplementation corresponds to a fourfold energy gain.

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 [48]. 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 and 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

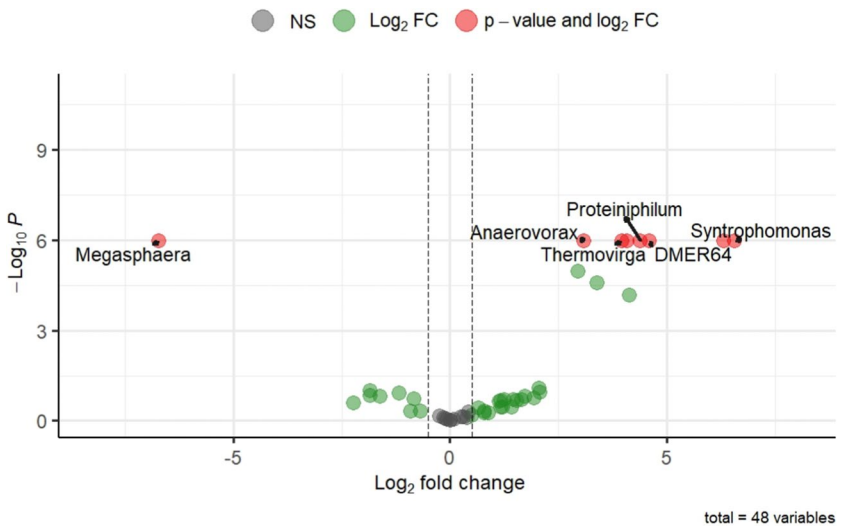

Fig. 5 Volcano plot for SBR in the fermentative and methanogenic stage

in the last operation cycles. This genus has been previously reported in changes in hydrogen metabolism to propionate production from succinate [49]. 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.

Figure 4 shows that contrasting microbial community structures were observed among acidogenic and methanogenic reactors. The distribution profile of ASV affiliated with *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. These results are shown in Fig. 5. *Proteiniphilum* spp. is a facultative anaerobic bacteria presumably generating acetic and propionic acids as main fermentation products [49]. *Thermovirga* spp. has been previously identified as a sulfate/Fe(III)-respiring gene with the ability to proceed with acetate oxidation [50]. 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.

This research was focused on TM effects in a two-stage system. However, TM effects 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 important to study the activity of the main metalloenzymes involved in methane synthesis in batch and SBR. In this way, it could be possible to detect if the addition of trace metals exerts a direct effect on the increase in enzymatic activity, or if other physicochemical factors could limit biogas production.

Conclusions

The results showed that adding 0.25 mg/L of Ni^{2+} and 334 mg/L of Fe^{2+} increased the hydrogen yields in batch. However, TM supplementation decreased productivity in the acidogenic reactor (first stage). The above was related to a high VS removal detected after each TM addition. Regarding the second stage, the undiluted AE caused the fast decay of the methanogenic reactor. Nevertheless, when the reactor was fed with the AE enriched with TM, the methane yield increased, and it was possible to maintain the stability of the process, avoiding inhibition. The metals changed the composition of the microbial communities in each stage.

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Data Availability Data available on request from the authors. The data of this study are available from the corresponding author, upon reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

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