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Effects of Magnetite (Fe_3O_4) as an Electrical Conductor of Direct Interspecies Electron Transfer on Methane Production from Food Wastewater in a Plug Flow Reactor

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Abstract: This study was conducted in order to examine the impact of magnetite (Fe_3O_4), a conductive material capable of promoting direct interspecies electron transfer (DIET) among microorganisms, on the efficiency of anaerobic digestion in a plug flow reactor (PFR) using food wastewater (FW) as the substrate. The effects of recovering and replenishing magnetite discharged along with the digestate during continuous operation of the PFR were also evaluated. A PFR with a total volume of 17 L was utilized as the reactor for anaerobic digestion. The inoculum was obtained from Icheon Biogas Research Facility, which operated with a mixture of pig slurry and FW in a 7:3 (*w*/*w*) ratio. FW was used as the substrate (volatile solids (VS) content of 85,865 mg-VS/L). The PFR was set for operation at 39 °C, and after a stabilization period of approximately 82 days, the hydraulic retention time (HRT) was set at 40 days. The study was conducted in three stages: stage 1 (83~122 days), stage 2 (123~162 days), and stage 3 (163~202 days). For the maintenance of an organic loading rate of 2.12 kg-VS/m^3/d, 0.3 L/d of substrate was added every 24 h, and analysis of an equal amount of discharged digestate was performed. The experimental treatments included a control without the addition of magnetite after the stabilization period, treatment (T1) with addition of magnetite (20 mM in digestate) and subsequent recovery and replenishment of magnetite on the discharge of digestate, and treatment (T2) with addition of magnetite (20 mM) without the replenishment of magnetite. Analytical parameters included the characteristics of the discharged digestate (pH, NH_4^+-N, chemical oxygen demand (COD_{Cr}), total volatile fatty acids (TVFAs), and alkalinity), and methane production (M_p). During the period of operation of the PFR after the stabilization period, no significant differences in pH and NH_4^+-N, based on the recovery and replenishment of magnetite, were observed, and a stably functioning PFR was observed. However, in stage 2, due to the increased degradation of organic matter caused by DIET, the COD_{Cr} of T1 and T2 decreased by 9.42% compared with the control. In stage 3, the magnetite content in the reactor in T2 decreased by a maximum of 9.42% compared to T1. In stage 3, the M_p for T2 was similar to that of the control, with a maximum discharge of magnetite of 3.06%, and the M_p decreased by 5.40% compared to T1. Regarding the ratio of methanogens in the community, the results of an analysis of the digestate from stage 3 showed an increase in the community of acetotrophic methanogens, specifically *Methanosarcina*. The findings of this study confirm that DIET was effectively promoted by maintaining the concentration of 20 mM magnetite in the PFR while using FW as a substrate.

Keywords: magnetite; food wastewater; direct interspecies electron transfer; plug flow reactor; anaerobic digestion

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1. Introduction

Anaerobic digestion (AD) is a biological conversion process that occurs under anaerobic conditions, which involves the sequential transformation and conversion of organic matter to biogas, including CH_4, by anaerobic microorganisms through hydrolysis, acidogenesis, acetogenesis, and methanogenesis [1]. In conventional AD processes, oxidation-reduction mediators, such as H_2 and formate, are required for indirect interspecies electron transfer (IIET), for the transfer of electrons between acetogens and methanogens using their nutritional symbiotic relationship. However, for substrates with high organic loading rates and organic acid content, imbalances in the rates of each transformation stage and a reduction in pH due to acid accumulation can inhibit anaerobic microbial activity, leading to the decreased efficiency of AD [2].

Recent studies have reported that the efficiency of AD can be enhanced by promoting direct interspecies electron transfer (DIET) for substrates with high organic loading and organic acid content, through an improvement in degradation rates and the prevention of accumulation of organic acid [3–5]. Unlike IIET, DIET does not require electron carriers and involves the introduction of conductive materials into anaerobic digesters to enhance the efficiency of AD through direct electron transfer reactions among anaerobic microorganisms [2]. Materials capable of promoting DIET include iron-based conductive substances (magnetite, hematite, goethite, ferrihydrite, etc.) and carbon-based materials (granular activated carbon, biochar, carbon cloth, graphite, etc.). Magnetism is a characteristic of magnetite (Fe_3O_4), an iron-based conductive material, which can enable easy recovery during the discharge process of anaerobic digestate. Magnetite's introduction into anaerobic digesters has been reported to enhance the efficiency of DIET by anaerobic microorganisms, due to its high conductivity [6–8]. AD studies involving the addition of conductive materials reported that the efficiency of AD varied depending on factors including the type of substrate, such as FW, livestock manure, and sewage sludge, the type of conductive material, and the input concentration. According to some reports, at certain concentrations or higher, excessive acceleration of the hydrolysis, acidogenesis, and acetogenesis phases can lead to inhibited production of CH_4 [9–11]. In AD using FW, some studies have reported that, due to the high concentration of organic matter, rapid degradation by anaerobic microorganisms can lead to excessive accumulation of total volatile fatty acids (TVFAs), resulting in a sharp decrease in pH [12,13]. Methanogens, which play a critical role in the production of methane during AD, include acetotrophic methanogens (AMs) and hydrogenotrophic methanogens (HMs), whose community composition can vary based on environmental factors (pH, temperature, hydraulic retention time, and substrate) [14,15]. In AD, *Methanimicrococcus* spp. belongs to the HMs; *Methanosarcina* spp. is known for its versatility, but within the context of anaerobic digestions, it is commonly referred to as belonging to the AMs. Jung et al. [16] reported that the addition of magnetite, at a concentration of 20 mM in anaerobic digestion using sewage sludge, resulted in the rapid production of CH_4 caused by the DIET effect of methanogens, leading to a significant dominance of the HM community. According to Baek et al. [17], continuous supplementation of magnetite is necessary during operation of a continuous anaerobic reactor with a substrate containing high concentrations of organic matter. In addition, Lee et al. [2] reported that introduction of FW as a substrate increased the rate of CH_4 generation with the addition of magnetite, at concentrations exceeding 20 mM, due to the effect of DIET. It was believed that the DIET effect would vary due to the variation in the content caused by the discharge of magnetite from an anaerobic digester.

Therefore, in this study, a plug flow reactor (PFR) was employed, and FW was used as the substrate, and analysis of the effects of DIET, based on the recovery and supplementation of conductive material, specifically 20 mM magnetite, in relation to the characteristics of anaerobic digestate, daily methane production (M_p), and the composition of the methanogen community, was performed. The objective of this study was to determine whether magnetite can exert a DIET effect when high-concentration organic matter is used as the substrate in the operation of a PFR.

2. Materials and Methods

2.1. Inoculum and Food Wastewater

The inoculum used in this study was collected from a mesophilic (38 °C) anaerobic digester with a capacity of 20 m^3/day, located in Icheon, Gyeonggi-do, Republic of Korea, where pig slurry and FW are treated in a 7:3 (*w*/*w*) ratio. The collected inoculum was subjected to a two-week mesophilic (38 °C) anaerobic digestion process to eliminate any remaining organic matter and gases. After this digestion, the physicochemical characteristics were analyzed (Table 1). The 300 L of FW utilized in this study was obtained from a food waste treatment facility located in Icheon. The substrate was divided into 20 L portions, stored at −20 °C, and each portion was thawed at 4 °C each time it was used. The physicochemical characteristics of the FW are shown in Table 1.

Table 1. Chemical composition of inoculum and food wastewater.

Parameter		Inoculum	Food Wastewater
pH	-	7.97 (±0.00)	3.43 (±0.15)
TS [(1)]	mg/L	42,900 (±299)	100,721 (±2263)
VS [(2)]	mg/L	24,250 (±111)	85,865 (±1992)
TKN [(3)]	mg/L	10,581 (±325)	14,856 (±1423)
NH_4^+-N [(4)]	mg/L	8631 (±68)	383 (±96)
COD_{Cr} [(5)]	mg/L	31,675 (±212)	177,273 (±4775)
$SCOD_{Cr}$ [(6)]	mg/L	22,360 (±294)	117,315 (±3671)
Alkalinity	(as $CaCO_3$)	17,200 (±164)	-
TVFAs [(7)]	(as acetate)	1011 (±4)	10,397 (±1550)

[(1)] Total solid, [(2)] volatile solid, [(3)] total Kjeldahl nitrogen, [(4)] ammonium nitrogen, [(5)] chemical oxygen demand, [(6)] soluble chemical oxygen demand, [(7)] total volatile fatty acids.

2.2. Continuous Anaerobic Digestion

A depiction of the continuous reactor used in this study is shown in Figure 1. The operational phases were designated as the stabilization period (~82 days), stage 1 (83~122 days), stage 2 (123~162 days), and stage 3 (163~202 days). The total volume of the PFR was 17 L. Three PRFs were employed in the study. Initially, 12 L of inoculum was introduced, and starting from the operational commencement date, FW was added daily while an equal amount of digestate was discharged and used for analysis. The pH of the inoculum was 7.97, the FW input was 300 mL/day at the same time every day, and the organic loading rate (OLR) was adjusted to 2.12 kg-VS/m^3/day. The conductive material for enhancement of DIET, magnetite powder (Samchun, particle diameter below 5 μm, Korea, CAS NO: 1317-61-9) was added at a concentration of 20 mM (4.63 g/L) based on effective volume after the stabilization period. The experimental treatments included a control, T1 (addition of 20 mM magnetite with recovery and replenishment), and T2 (addition of 20 mM magnetite without replenishment). A magnet (Umagnet, Seoul, Korea) was used for recovery of the magnetite discharged along with the digestate, followed by washing three times with distilled water, drying, and weighing. Biogas was captured in a 30 L gas bag (Dong Bang Hitech, Seoul, Korea) and the measurement of gas production was performed using a flow meter (Kemik, Seoul, Korea). The volume of biogas generated was standardized by converting it under standard conditions (0 °C, 1 atm), as shown in Equation (1). In this equation, $V_{dry\ gas}$ represents the volume of dry gas under standard conditions (0 °C, 1 atm), T indicates the operating temperature of the reactor, $V_{wet\ gas\ at\ T°C}$ indicates the volume of humid gas at the reactor's operating temperature (38 °C), P indicates the atmospheric pressure at the time of gas volume measurement, and PT indicates the saturated water vapor pressure at T°C (mmHg). In this study, P was 760 mmHg, and calculation of P_T was based on the saturated water vapor pressure of water at 38 °C [18].

$$V_{dry\ gas} = V_{wet\ gas\ at\ T°C} \times \frac{273}{(273+T)} \times \frac{(P-P_T)}{760} \quad (1)$$

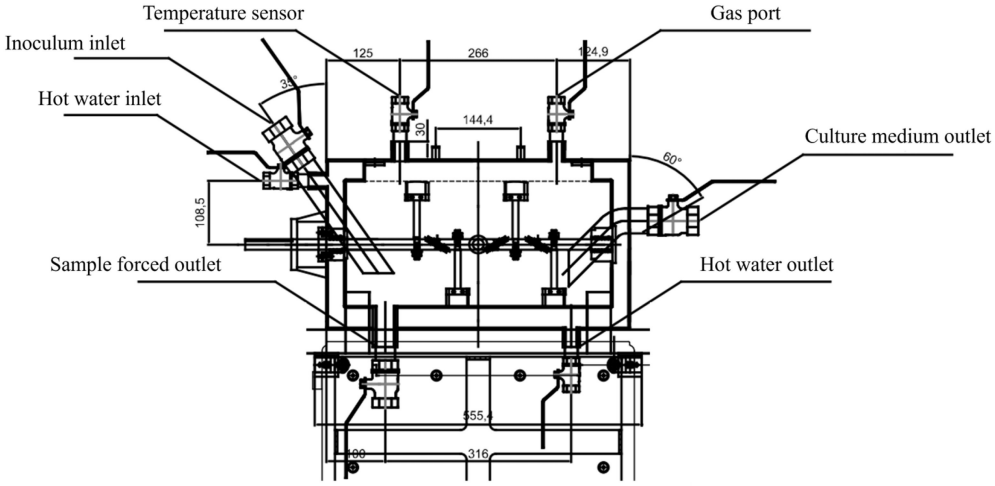

Figure 1. A schematic diagram of plug flow reactor operation. (The sample comes out from the culture medium outlet).

2.3. Analysis

The analysis of gas composition of biogas was performed using a gas chromatography (PerkinElmer, Boston, MA, USA) equipped with a thermal conductivity detector (TCD). A HayesepQ packed column (3 mm × 3 m, 80~100 mesh size) was used, and high-purity argon (Ar) gas was used as the carrier gas, with a flow rate of 30 mL/min. The analysis was performed under the following operating conditions: injector temperature at 150 °C, column oven temperature at 90 °C, and detector temperature at 150 °C [19]. Analyses of total solids (TS), volatile solids (VS), chemical oxygen demand (COD_{Cr}), soluble chemical oxygen demand ($SCOD_{Cr}$), total Kjeldahl nitrogen (TKN), ammonium nitrogen (NH_4^+-N), alkalinity, total volatile fatty acids (TVFAs), and others were performed according to standard analytical methods [20].

2.4. DNA Extraction

Extraction of DNA was performed using a DNA extraction kit (Biofact, Seoul, Korea). For the extraction procedure, 200 µL of a solution containing 1% sodium dodecyl sulfate and 1% ethylenediaminetetraacetic acid (EDTA), along with 2 µL of lysozyme, was added to the microbial samples collected from digestate, and the mixture was incubated at 37 °C on a heat block for 30 min. A solution (200 µL) containing 5% Tris buffer, 5% Triton buffer, and 1% ammonium chloride was subsequently added, along with 5 µL of proteinase and 2 µL of RNase A. The mixture was heat-treated at 50 °C for 40 min, followed by further incubation at 70 °C for 15 min. Following this step, centrifugation (13,000× *g*, 10 min) was performed, with the addition of 200 µL of binding buffer to the upper layer. All liquids were centrifuged using a spin column tube (7000× *g*, 1 min), followed by two washes using wash buffer. The DNA was then diluted with DNA hydration solution (40 µL) and centrifugation was performed (13,000× *g*, 10 min) in order to obtain the final DNA.

2.5. Library Construction and Sequencing

The sequencing libraries were prepared according to the Illumina 16S Metagenomic. Sequencing library protocols were used for amplification of the V3 and V4 regions. The input gDNA 5 ng was amplified using PCR with 5x reaction buffer, 1 mM of dNTP mix, 500 nM each of the universal F/R PCR primer, and Herculase II fusion DNA polymerase (Agilent Technologies, Seattle, WA, USA). The cycle condition for the first PCR was 3 min at 95 °C for heat activation, and 25 cycles of 30 s at 95 °C, 30 s at 63 °C and 30 s at 72 °C, followed by a 5 min final extension at 72 °C. The universal primer pair with Illumina adapter overhang sequences used for the first amplifications were as follows:

16S 787F Amplicon PCR Forward Primer: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATTAGATACCCSBGTAGTCC

16S 1059R Amplicon PCR Reverse Primer: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCCATGCACCWCCTCT

The first PCR product was purified using AMPure beads (Agencourt Bioscience, Beverly, MA, USA), followed by amplification of 10 µL of the first PCR product, using PCR, for construction of the final library containing the index, using NexteraXT Indexed Primer. The cycle condition for the second PCR was the same as that used in the first PCR condition, except for 10 cycles. The PCR product was purified using AMPure beads. Quantification of the final purified product was then performed using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and qualification was performed using the TapeStation D1000 ScreenTape (Agilent Technologies). Sequencing was then performed using the MiSeq™ platform (Illumina, San Diego, CA, USA).

2.6. Statistical Analysis

Statistical analysis of the experimental results was performed using the GLM (general linear model) procedure from the SAS® software package (ver. 9.4, SAS Institute Inc., Cary NC, USA). Duncan's multiple range test was performed to determine the significance ($p < 0.05$) of differences in means among treatments [21].

3. Results and Discussion

3.1. Changes in the Composition of Anaerobic Digestate and Methane Production

The results of the analysis of the digestate released during the operation of the continuous anaerobic digestion reactor, PFR, from stage 1 to stage 3, are shown in Table 2 and Figure 2. Throughout all stages, the pH within the PFR remained within an appropriate range of 7.71–7.80 (pH 7.7–8.2) [22]. The concentrations of NH_4^+-N, ranging from 3002 to 3488 mg/L, were consistent and did not reach levels that could affect the performance of the PFR [23]. The TVFAs/alkalinity within the PFR were maintained at levels between 0.04 and 0.11, well below the maximum value of 0.4, ensuring stable organic loading [24]. In stage 2, the addition of magnetite resulted in a significant reduction ($p < 0.05$) in COD_{Cr} for both T1 (3.02%) and T2 (4.50%) compared with the control, as well as a decrease in TVFAs by 35.33% and 35.40%, respectively. In stage 3, notable differences in the characteristics of the digestate were observed among treatment groups, and significantly higher COD_{Cr} (27,309 mg/L) and TVFAs (1418 mg/L) were observed for the control ($p < 0.05$). Compared with the control, T1 and T2 showed a reduction in COD_{Cr}, of 8.58% and 9.42% and 46.97% and 47.11% in TVFAs, respectively. The findings of one study indicated that operational time can be reduced, and that degradation of organic matter can be enhanced, with the use of the DIET effect, particularly at higher organic loading rates, within an anaerobic digestion reactor [25]. In this study, during stages 2 and 3, it was observed that, regardless of magnetite recovery and supplementation, the rate of organic degradation of substrate increased, leading to a reduction in COD_{Cr}. While the concentration of TVFAs in the control group increased by 7.37% and 10.01% in stages 2 and 3, respectively, compared to stage 1, reductions in TVFAs of 49.75% and 32.83%, respectively, were observed for T1 and T2 in stage 2, and 32.83% and 43.65%, respectively, in stage 3. This paradoxical reduction in TVFAs, despite the expected increase due to the DIET effect of magnetite, can be attributed to the faster conversion rates of TVFAs into CH_4 and CO_2 [6].

Table 2. Chemical characteristics of anaerobic digestate and daily methane production from addition of magnetite to plug flow reactor incubation at each stage.

Operation Step		pH	NH_4^+-N [(1)]	TVFAs [(2)]	COD_{Cr} [(3)]	FOS/TAC [(4)]	M_r [(5)]	M_p [(6)]
		(-)	(mg/L)	(mg/L)	(mg/L)	(-)	(%)	(L/day)
Stage 1 [(7)]	Control	7.71	3134	1289 [b]	27,004	0.09	-	10.92 [b]
	T1 [(10)]	7.73	3202	1781 [a]	28,363	0.11	0	11.22 [a]
	T2 [(11)]	7.74	3225	1331 [b]	28,262	0.08	0.97	11.21 [a]
	SEM	0.02	102	82	55	-	-	0.07
	p-value	<0.05	<0.05	<0.05	<0.05	-	-	<0.05
Stage 2 [(8)]	Control	7.79	3316	1384 [a]	27,504 [a]	0.10	-	10.50 [c]
	T1	7.80	3488	895 [b]	26,266 [b]	0.07	0	11.09 [a]
	T2	7.80	3218	894 [b]	26,673 [b]	0.06	1.97	10.74 [b]
	SEM	0.01	161	111	427	-	-	0.04
	p-value	<0.05	<0.05	<0.05	<0.05	-	-	<0.05
Stage 3 [(9)]	Control	7.80	3130 [ab]	1418 [a]	27,309 [a]	0.09	-	10.25 [b]
	T1	7.80	3002 [b]	752 [b]	24,965 [b]	0.05	0	10.92 [a]
	T2	7.79	3177 [a]	750 [b]	24,736 [b]	0.04	3.06	10.33 [b]
	SEM	0.01	53	181	586	-	-	0.06
	p-value	<0.05	<0.05	<0.05	<0.05	-	-	<0.05

[ab] Means with a different letter differed significantly between treatments ($p < 0.05$). SEM = standard error of the mean. [(1)] Ammonium nitrogen, [(2)] total volatile fatty acids, [(3)] chemical oxygen demand, [(4)] volatile organic acids/total inorganic carbon (alkalinity/TVFAs), [(5)] magnetite recovery rate, [(6)] daily methane production, [(7)] fermentation time during a period of 40 days from the 83rd to the 122nd day after operation of the plug flow reactor, [(8)] fermentation time during a period of 40 days from the 123rd to the 162nd day after operation of the plug flow reactor, [(9)] fermentation time during a period of 40 days from the 163rd to the 202nd day after operation of the plug flow reactor, [(10)] maintenance replenishment after addition of 20 mM magnetite compared to digestate [(11)] only 20 mM magnetite added.

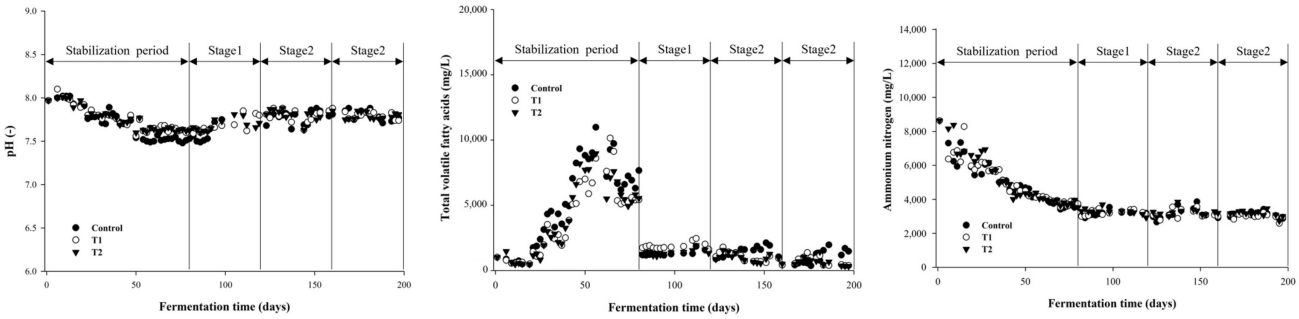

Figure 2. The effects of addition of magnetite on pH, TVFAs, and ammonium nitrogen in the plug flow reactor (T1: maintenance replenishment after addition of 20 mM magnetite compared to digestates; T2: only 20 mM magnetite added).

The analysis of M_p for each stage of operation of the PFR after the stabilization period is shown in Table 2. During stage 1, both T1 and T2 showed significantly higher M_p than the control ($p < 0.05$). In stage 1, the quantity of magnetite released from the reactor in T2 was negligible, accounting for only 0.97%. As a result, no substantial disparity in M_p was observed between T1 and T2, which showed increases of 2.67% and 2.59%, respectively, compared with the control group ($p < 0.05$). However, during stage 2, due to an increased loss of magnetite in T2 of 1.97%, its M_p was 3.19% lower than that of T1 and 2.23% higher compared with the control ($p < 0.05$). In stage 3, T1 showed the highest level of M_p at 10.92 L/day, an increase of 6.14% compared with the control ($p < 0.05$). However, the escalated loss of magnetite, totaling 3.06%, resulted in a 5.4% lower level of M_p compared to T1 and no significant difference from the control. Studies reported by Jing et al. [26] and Yin et al. [27] have suggested that enhancement of M_p can be achieved by the

addition of magnetite during anaerobic digestion using glucose as a substrate. Kato et al. [6] reported increased M_p with the addition of magnetite (20 mM) in acetate-fed co-cultures of two anaerobic microorganisms compared with the control group. Similarly, Lee et al. [2] reported an increase in the production rate of CH_4, CH_4 yield, and substrate degradation rate when evaluating the effects of magnetite at levels ranging from 5 mM to 100 mM in pig slurry and FW substrate-fed anaerobic digestion; the increase was observed above a concentration of 20 mM. In comparison with the studies mentioned above, in stage 1, it was observed that an increase in M_p occurred regardless of magnetite loss and supplementation, compared with the untreated group. However, in stage 2, as the loss of magnetite increased in T2, a difference between T1 and T2 began to emerge. Subsequently, in stage 3, as the loss of magnetite in T2 reached 3.06%, M_p decreased to a level comparable with that of the control. Lee et al. [28] reported that, in continuous AD using high-concentration organic matter and supplemented with magnetite, consistent addition of magnetite is necessary for the maintenance of M_p. In the results of this study, during operation of the PFR, T2—where the loss of magnetite was not compensated for—showed a maximum decrease in magnetite content of 3.06% within the reactor compared to T1. Consequently, the concentration of magnetite within the PFR dropped to 19 mM. However, even at this level, its influence on M_p continued.

3.2. Microbial Community

During the 40-day period of operation of the PFR in stage 3, samples were collected by combining the anaerobic digestates from each treatment group. Subsequently, analyses of the differences in the methanogens community and bacterial community were performed separately. The microbial community was classified according to "Phylum" and "Genus". In this study, the clusters representing less than 1.00% of the microbial community, in all treatment groups, were categorized as "other". The results of analysis for the methanogens community in the anaerobic digestates for each treatment group are shown in Table 3, and the results of the analysis of the bacterial community are shown in Table 4. Within the PFR, at the phylum level of the methanogen community, a prominent presence of *Candidatus Thermoplasmatota* and *Euryarchaeota* was observed. Within the PFR, for the genus *Candidatus Thermoplasmatota*, only *Methanomassiliicoccus* was identified. *Methanomassiliicoccus*, a hydrogenotrophic methanogen classified as a HM, showed lower percentages in T1 (1.93%) and T2 (1.98%) compared with the control (9.78%). At the phylum level within the PFR, *Euryarchaeota* showed higher community proportions in T2 (97.65%) and T1 (97.20%) compared with the control (89.93%). At the genus level of *Euryarchaeota*, *Methanoculleus*, a hydrogenotrophic methanogen, showed the highest percentage in the control (87.99%), while it was lower in T1 (38.89%). In the same phylum, *Methanosarcina*, classified as an an AM, was not observed in the control, but showed higher community proportions in T1 (53.32%) than in T2 (23.82%). Baek et al. [25] previously reported a higher abundance of HMs (e.g., *Methanomassiliicoccus*) without the addition of magnetite in continuous anaerobic digestion, in agreement with the results of this study. Jang et al. [29] reported that in an anaerobic digestion reactor fed with FW, the analysis of the methanogen community showed that initially, AMs dominated at 81.3%. However, with the progression of time, the dominance of AMs showed a gradual decline, and an increase in HMs was observed. In this regard, Kim et al. [30] and Jang et al. [31] reported that in continuous anaerobic digestion, a transition from AMs to HMs occurs with the progression of time. In stage 3, where the anaerobic digestion time exceeded 80 days, except for in T1, the same results were observed for both the control and T2. In the results of this study, AMs were not observed in the control, while they were observed only in T1 and T2, where magnetite was added. In addition, as the concentration of magnetite in the reactor decreased, HMs showed an increase in T2 compared with AMs. Jung et al. [16] reported that when magnetite was utilized in anaerobic digestion, lower proportions were observed for the community of HMs, including *Methanoculleus*. Similarly, the results of this study showed that the *Methanoculleus* community showed the highest levels in the control, and among the treatments, higher

levels were observed for T2 compared to T1, where the concentration of magnetite was maintained. Lei et al. [32] reported an increase in the *Methanosarcina* community with the addition of magnetite to an anaerobic digestion reactor, potentially due to the enhancement of DIET from the direct transfer of electrons from conductive materials. However, the findings of this study indicated that the *Methanosarcina* community decreased in T2, where the concentration of magnetite decreased compared to T1. No significant differences in the bacterial community within the anaerobic digestate of the PFR was observed among the treatment groups. Findings reported by Zheng et al. [33] demonstrated that use of high-concentration organic matter as a substrate and magnetite to confirm the DIET effect did not impact the structure of the microbial community.

Table 3. Taxonomic composition ratio of methanogens at the phylum and genus levels in anaerobic digestion with addition of magnetite.

Phylum	Genus	Metabolic [1]	(%)		
			Control	T1 [4]	T2 [5]
Candidatus Thermoplasmatota	*Methanomassiliicoccus*	HMs [2]	9.78	1.93	1.98
	Methanoculleus	HMs	87.99	38.89	72.37
Euryarchaeota	*Methanimicrococcus*	HMs	1.94	5.44	1.01
	Methanosarcina	AMs [3]	0.00	53.32	23.82
Others [6]		-	0.29	0.42	0.83

[1] Metabolic pathway for methanogenesis [2] hydrogenotrophic methanogens, [3] acetotrophic methanogens, [4] maintenance replenishment after addition of 20 mM magnetite compared to substrate, [5] only 20 mM magnetite added, [6] less than 1% methanogens.

Table 4. Taxonomic composition ratio of bacteria at the phylum and genus levels in anaerobic digestion with addition of magnetite.

Phylum	Genus	(%)		
		Control	T1 [1]	T2 [2]
Atribacterota	*Atribacter*	64.02	66.39	65.54
Bacillota	*Capillibacterium*	5.73	4.53	5.54
	Thermanaeromonas	3.59	3.33	3.01
	Syntrophaceticus	6.89	5.59	5.09
	Keratinibaculum	2.42	2.54	2.36
Others [3]		17.35	17.62	18.46

[1] Maintenance replenishment after addition of 20 mM magnetite compared to substrate, [2] only 20 mM magnetite added, [3] less than 1% methanogens.

4. Conclusions

The purpose of this study was to examine the enhancing effect of DIET through recovery and supplementation after addition of 20 mM magnetite, a conductive material, to a PFR using FW as the substrate. The objective was to determine the impact of the promotion of DIET on the characteristics of the anaerobic digestate, M_p, and MC. The stabilization period was conducted until pH, NH_4^+-N, and the level of TVFAs became consistent. Throughout operation of the PFR, pH and NH_4^+-N were maintained within an appropriate range regardless of magnetite addition or replenishment. TVFAs/alkalinity remained between 0.04 and 0.11, ensuring stable operation. However, after stage 2, the increased rate of organic degradation caused by DIET led to a reduction in COD_{Cr} of 9.42% and a reduction in TVFAs of 47.11% for both T1 and T2 compared with the control. These results are thought to have affected M_p as a DIET promotion. T1, where the input quantity of magnetite into the PFR was maintained at 20 mM, consistently showed the highest level of M_p in all stages, indicating the positive effect of DIET. However, in T2, where magnetite was discharged along with the digestate, the level of M_p decreased as the period of operation of the PFR was extended. In stage 3, there was a magnetite loss

of 3.06%, and the levels of M_p observed for T2 were similar to those of the control. In Stage 3, in the methanogen community, the *Methanosarcina* community, which benefits from DIET through conductive materials, was highest in T1. In T2, due to the reduced concentration of magnetite within the PFR and the diminishing effect of DIET promotion, the presence of methanogens within the *Methanosarcina* genus was observed, but at a lower level compared to T1. In addition, it is expected that TVFAs will significantly decrease compared to the control, due to the increase in the proportion of *Methoansarcina*, AMs, and the increased utilization of acetate.). The findings of this study confirm that the use of FW as a substrate in the PFR while maintaining the concentration of 20 mM magnetite, can enhance anaerobic digestion. The conduction of additional research will be needed for the evaluation of concentrations of magnetite exceeding 20 mM, at which M_p can be sustained without additional supplementation.

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