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Effects of long-term acclimatization on the optimum substrate mixture ratio and substrate to inoculum ratio in anaerobic codigestion of food waste and cow manure

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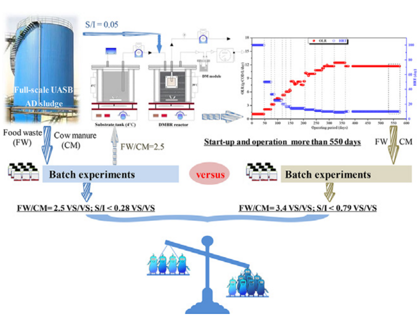
GRAPHICAL ABSTRACT



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ABSTRACT

The effects of long-term acclimatization on the optimum food waste to cow manure ratio (FW/CM) and substrate to inoculum ratio (S/I) in anaerobic codigestion with FW and CM were investigated by batch trials. For the unacclimated sludge, the highest CH_4 yields of 646.6 and 653.4 mL/g VS were achieved under the optimum FW/CM (2.5 VS/VS) and S/I (0.07 VS/VS) ratios, respectively. After more than 550 days of acclimatization, the optimum FW/CM and S/I of the acclimated sludge were improved to 3.4 and 0.68 VS/VS with more anaerobic digestion enzymes and lignocellulose, respectively. Based on high-throughput sequencing analysis, the microbial community structures of bacteria, fungi, and archaea were changed, which was the main reason for the change in the optimum FW/CM and S/I. Therefore, the FW/CM and S/I should be periodically optimized during the long-term operation of codigestion to improve the codigestion efficiency for biogas production.

1. Introduction

A large amount of fossil fuel is used for energy generation; fossil fuel not only is a challenge due to resource shortages but also increases global greenhouse gas emissions. Therefore, alternative renewable energy sources are attracting much attention. Food waste (FW) has been considered a promising feedstock for producing bioenergy or biofuel. Anaerobic digestion (AD) technology is an effective method for the optimization of FW management and bioenergy production (Xu et al., 2018). However, the monodigestion of FW can be inhibited at an

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Table 1
Physicochemical characteristics of CM, FW, and inoculum.

Parameter	CM	FW	Inoculum	
			Seed sludge	Acclimated sludge
TS (g/L)	113.7	82.3	41.6 (30.2)	23.9 (19.4)
VS (g/L)	95.9	81.2	18.1 (15.1)	18.0 (14.8)
TCOD (g/L)	64.6 ± 9.0	212.6 ± 5.5	24.7 (24.0)	22.5 (20.4)
SCOD (g/L)	11.7 ± 0.4	88.7 ± 1.6	0.3 (1.6)	1.4 (1.7)
pH	7.08	4.34	7.85 (7.75)	7.24 (7.29)
Protein (g/L)	3.80 ± 0.12	3.07 ± 0.03	0.01 (0.33)	0.72 (0.55)
Carbohydrate (g/L)	1.36 ± 0.02	82.1 ± 1.5	0.01 (0.11)	0.46 (0.30)
NH_4^+-N (mg/L)	376.5	312.2	89.8 (65.4)	1054.4 (2656.4)
Alkalinity (g $CaCO_3$/L)	13.5	0	13.0 (1.45)	3.3 (31.0)
Acetic acid (mg COD/L)	74.7	1980.5	21.2 (10.5)	32.3 (12.3)
Propionic acid (mg COD/L)	22.2	0	0 (0)	0 (0)
Butyric acid (mg COD/L)	6.9	0	0 (0)	28.9 (14.0)

Notes: the values in parentheses are the second inoculum values to determine the optimum S/I.

Table 2
Operating characteristics of batch trials.

Test	FW/CM	S/I
Seed sludge	FW/CM = 3.4, 2.5, 1.7, 0.8, 0.4, 0.3, 0.2 (S/I = 0.05)	S/I = 0.07, 0.14, 0.28, 0.56, 1.12 (FW/CM = 2.5)
Acclimated sludge*	FW/CM = 3.4; 2.5; 1.7; 0.8; 0.4; 0.3; 0.2 (S/I = 0.62)	S/I = 0.57, 0.68, 0.79, 0.91, 1.02, 1.13 (FW/CM = 3.4)

Note: * Start-up and acclimatization in a bioreactor operated with the FW/CM of 2.5 for over 550 days with a stepwise increase in the OLR from 0.6 to 7.81 g VS/L/day.

extremely high organic loading rate (OLR) due to an improper carbon-to-nitrogen (C/N) ratio, imbalanced nutrients, volatile fatty acid (VFA) accumulation, ammonia inhibition, and low buffer capacity (Xu et al., 2018; Zan and Hao, 2020). Among the various effective enhancement strategies that have been investigated, the codigestion of FW with other substrates, such as lignocellulosic biomass (Li et al., 2020), waste activated sludge (Tao et al., 2020) and animal manure (Ebner et al., 2016), exhibited promising advantages. Cow manure (CM) as a substrate contains protein, fat, cellulose, and lignin; however, the high content of nonbiodegradable and degradation-resistant substances in CM cannot be completely biodegraded into biofuels (Bi et al., 2020). Therefore, the codigestion of FW and CM could be a solution to avoid problems such as VFA accumulation and high ammonia concentrations to maintain a better nutrient balance and buffer capacity in the codigestion system.

To maintain the stability of the AD process, some parameters, such as sludge retention time (SRT), hydraulic retention time (HRT), and OLR, could be crucial. Moreover, the substrate mixing ratio (FW/CM) and substrate to inoculum ratio (S/I) also influenced CH_4 production in the codigestion with FW and CM (Xing et al., 2020a). A review on the thermophilic codigestion of FW and dairy cattle manure revealed that a mixture of 65.3% FW and 34.7% cattle manure can provide the highest CH_4 yield (Zarkadas et al., 2015). Muratçobanoğlu et al. (2020) reported that codigestion contributed to improving biogas production with the FW/CM of 2.0 (based on volatile solids, VS), which was consistent with the result reported by Zhang et al. (2013). Ebner et al. (2016) found that the optimal S/I of 2 VS/VS prevented biomass limiting kinetics. However, Xing et al. (2020a) reported an optimum FM/CM ratio of 2.5 VS/VS and an S/I of no more than 0.07 VS/VS, which were largely different from the results of the above studies, even with similar feedstock styles. The different characteristics of the substrate and inoculum may be the main reason for the different optimum substrate mixing ratios and S/I in previous studies. Therefore, the optimum substrate mixing ratio and S/I should be further compared and confirmed based on a previous study as well as the real substrate and inoculum for rapid and stable start-up codigestion. Although the optimum substrate mixing ratio and S/I for start-up codigestion have been determined, few studies have considered the long-term acclimatization effect of the optimum substrate mixing ratio and S/I on the codigestion system.

Thus, the main objectives of this study are to (1) optimize the FW/CM and S/I after more than 550 days of acclimatization under the initial optimum FW/CM before start-up codigestion with FW and CM; (2) compare the changes in the optimum FW/CM and S/I using the initial seed sludge and acclimated sludge; and (3) explain the reasons for the changes in the optimum FW/CM and S/I after acclimatization in the view of key AD enzymes, lignocellulase, and the microbial community.

2. Material and methods

2.1. Feedstock and inoculum

The FW was manually prepared based on the characteristics of FW in China (Li et al., 2018). CM was collected from a rural area near Xi'an, China. The inoculum used in this study included digestion sludge with and without acclimation by feeding feedstock, as shown in Table 1. The initial seed sludge was collected from a brewery wastewater treatment plant in Xi'an, China. After long-term operation of approximately 550 days, the acclimated digestion sludge was taken from a lab-scale mesophilic anaerobic dynamic membrane bioreactor (DMBR) as described in Section 2.2. The physicochemical characteristics of the feedstock and inoculum, including the initial seed sludge without acclimatization and the acclimated digestion sludge, are presented in Table 1.

2.2. Experimental procedures

Batch trials were conducted under mesophilic temperature of 39 °C. First, the monodigestion of FW and CM was carried out. Meanwhile, different substrate combination ratios were tested under mesophilic conditions at an S/I of 0.05 VS/VS. Based on the results from the above batch trials, the optimum FW/CM in the feedstock was determined. Then, the second batch trials were set up to further research the optimum S/I; five different S/I were tested with a constant amount of inoculum in all batches. All of these trials were conducted in duplicate. Samples were mixed for 3–5 min and then purged with nitrogen gas for

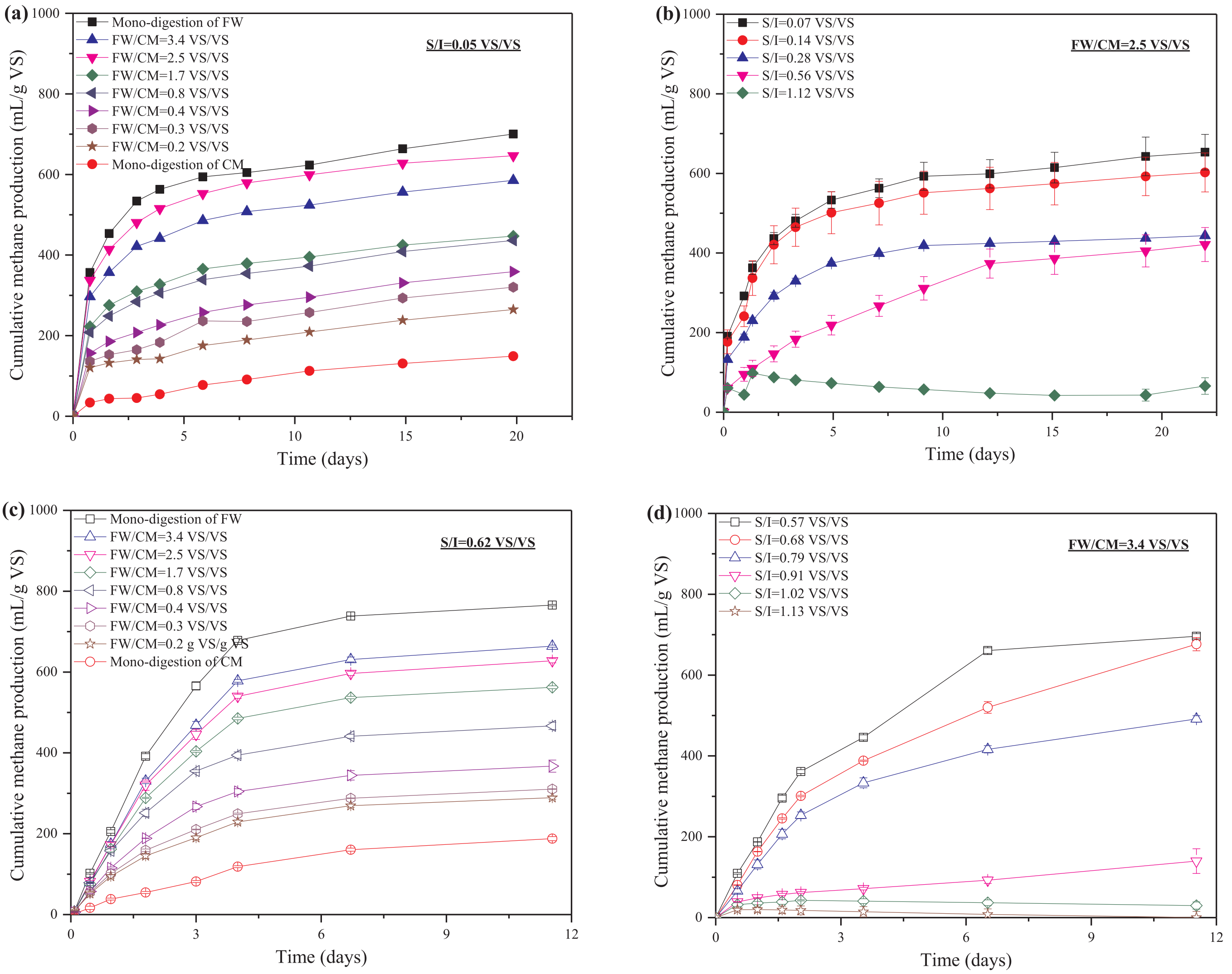

Fig. 1. Cumulative CH_4 production of anaerobic codigestion with FW and CM under different FW/CM and S/I (a-b) before and (c-d) after long-term acclimatization.

approximately 2 min.

After optimization of start-up conditions, the codigestion with FW and CM were conducted in a 0.7-liter semicontinuous DMBR under mesophilic temperature of 39 °C. The filtering surface area and pore size of the submerged filtration module were 14.4 cm^2 and 50 μm, respectively. After a stable start-up was achieved, the OLR was increased with decreasing the HRT during Day 1 to Day 312. Then, the DMBR was still operated under high-rate loading for more than 300 days. From Day 532 to Day 573, the discharge sludge of DMBR was collected and then used as acclimated sludge. To determine the optimum FW/CM in feedstock, batch mono- and codigestion trials were also established similar to the initial batch trials. Based on the results from the above batch test and DMRB performance, the S/I was further investigated at higher values, as described in Table 2. In addition, samples were also collected from the initial seed sludge and the acclimated sludge to characterize the enzyme content and the microbial community.

2.3. Analytical methods

The biogas production was analyzed using a glass syringe. The composition of the various biogases was measured using a gas chromatograph (GC7900, Tianmei, China) equipped with a thermal conductivity detector (TCD) and a 2-m carbon molecular sieve TDX-01 column. The pH values were monitored using a portable pH meter (Horiba, Kyoto, Japan). The levels of soluble chemical oxygen demand (SCOD), total chemical oxygen demand (TCOD), total solids (TS), VS, and NH_4^+-N were analyzed based on standard methods (APHA, 2005). Proteins and carbohydrates were measured by the Lowry-Folin method and H_2SO_4/phenol oxidation, respectively (Lowry et al., 1951; DuBois et al., 1956). Volatile fatty acids (VFAs) were measured using a PANNO gas chromatograph. Alkalinity was assayed according to the research by Kafle and Kim (2011). The conversion efficiencies of AD reaction ratios (hydrolysis, acidogensis, acetogenesis, and methanogenesis) were calculated based on the COD balance (Shofie et al., 2015). AD enzymes and lignocellulase were measured by enzyme-linked immunosorbent assay (ELISA) kits (MSKBIO, Wuhan, China) according to the manual instructions. Microbial community analysis was determined via high-throughput sequencing technology as proposed by Xing, et al. (2020b).

3. Results and discussion

3.1. Changes in the optimized FW/CM and S/I through prolonged acclimation

3.1.1. Cumulative CH_4 production and enhanced CH_4 yield percentage

Anaerobic batch experiments were conducted to compare the CH_4 production of FW and CM in mono- and codigestion (Fig. 1). The trial results regarding CH_4 production were fitted with the modified Gompertz model and first-order model to examine the suitability of different FW/CM and S/I with respect to CH_4 generation. The cumulative CH_4

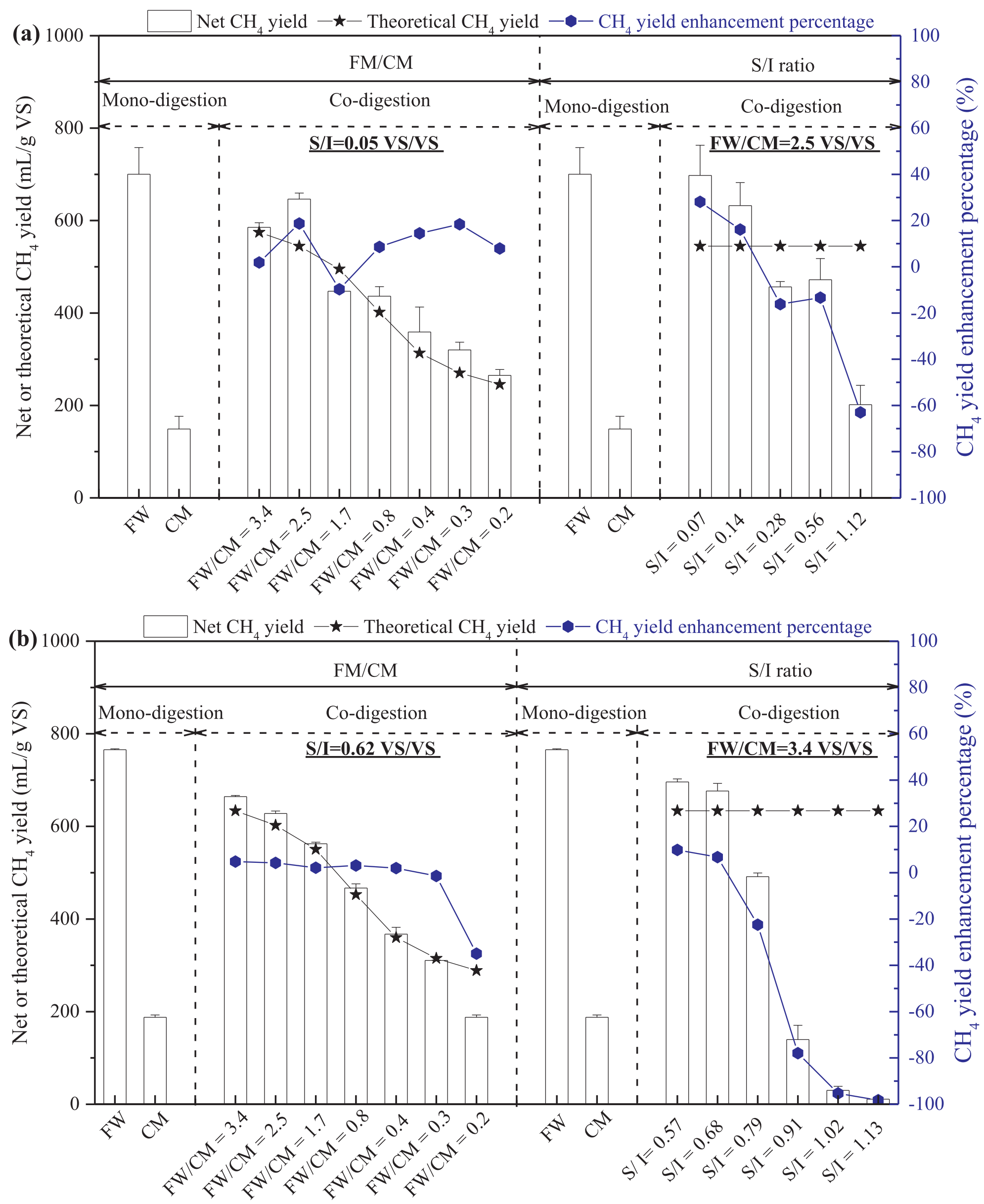

Fig. 2. The net and theoretical CH_4 yield and enhanced CH_4 yield percentage of anaerobic codigestion with FW and CM values under different FW/CM and S/I (a) before and (b) after long-term acclimatization.

production of seven FW/CM was measured with inoculum sludge with or without long-term acclimatization, as described in Fig. 1a and c. Before the digester start-up, the highest cumulative CH_4 production of 646.6 mL/g VS was achieved at the FW/CM of 2.5 VS/VS, and the corresponding CH_4 yield under this condition was enhanced by approximately 18.7% (Fig. 2a). The cumulative CH_4 production was well expressed by the modified Gompertz model and first-order model, as shown in Table 3 and Table 4. During the batch trials with initial seed sludge without acclimatization, the maximum P_0 of 581 mL/g VS and R_{max} of 254 mL/day/g VS were also observed at the FW/CM of 2.5 VS/VS (Table 3). These results indicated that the optimum FW/CM of 2.5 VS/VS can be used to start up codigestion with FW and CM to maximally improve CH_4 production and CH_4 yield. After long-term acclimatization, the maximum cumulative CH_4 production of 664 mL/g VS was realized at the FW/CM of 3.4 VS/VS, which was further increased by approximately 2.7% compared with the maximum cumulative CH_4 production of 647 mL/g VS at the FW/CM of 2.5 VS/VS. Meanwhile, the corresponding P_0 value also increased from 415 to 686 mL CH_4/g VS, as described in Table 3 and Table 4. These results revealed that the optimum FW/CM for codigestion was not constant during the long-term operation. The relative amount of CM could be further reduced to achieve better codigestion efficiency after long-term acclimatization. As shown in Table 6, the optimum FW/CM used in previous studies were evidently different with different inoculum sludge. Zhang et al. (2013) reported that a higher CH_4 yield of 388 mL/g VS was realized at the FW/CM of 2 VS/VS compared to the CH_4 yield at the FW/CM of 4 VS/VS in batch trials using anaerobically treated activated sludge as inoculum sludge. Thus, the optimum FW/CM should be optimized before start-up codigestion with FW and CM with different inoculum and substrate sources.

After determining the optimum FW/CM, the cumulative CH_4 production at different S/I with seed sludge and acclimated sludge was determined, as shown in Fig. 1b and d, respectively. For the seed sludge without acclimated batch trials, the cumulative CH_4 production decreased from 653.4 to 65.8 mL/g VS as the S/I increased (Fig. 1b). The cumulative CH_4 production at the S/I of 0.07–0.56 VS/VS was well expressed (R^2 greater than 0.9300) by the modified Gompertz model and first-order model (Table 3). Compared with the S/I of 0.07–0.56

Table 3
Kinetic parameters of CH_4 production with respect to different FW/CM and S/I obtained from the modified Gompertz model and first-order model using seed sludge without acclimatization.

Samples	FW/CM (VS/VS)	S/I (VS/VS)	Modified Gompertz model				First order model		
			P_0 (mL/g VS)	R_{max} (mL/day/g VS)	t_0 (d)	R^2	P_0 (mL/g VS)	k (day^{-1})	R^2
FW	/	0.04	616.0	318.9	0	0.9109	626.7	0.848	0.9562
CM	0	0.05	154.5	10.2	0	0.9612	160.4	0.117	0.9585
FW/CM	3.4	0.05	516.3	197.6	0	0.8786	522.2	0.739	0.93427
	2.5	0.05	581.3	253.5	0	0.8955	590.0	0.783	0.9480
	1.7	0.05	389.9	144.0	0	0.8626	394.3	0.727	0.9217
	0.8	0.05	379.8	99.3	0	0.8436	378.0	0.630	0.9013
	0.4	0.05	321.1	47.4	0	0.8344	309.0	0.460	0.8726
	0.3	0.05	294.2	33.6	0	0.8438	280.4	0.360	0.8593
	0.2	0.05	249.4	21.8	0	0.7970	223.9	0.384	0.7916
S/I	2.5	0.07	602.4	166.7	0	0.9300	601.7	0.635	0.9361
	2.5	0.14	560.4	167.9	0	0.9427	562.5	0.628	0.9471
	2.5	0.28	422.6	104.6	0	0.9474	422.2	0.5605	0.9470
	2.5	0.56	415.3	35.2	0	0.9758	421.1	0.166	0.9737
	2.5	1.12	/	/	/	/	63.93	17.39	0.4563

Note: "/" means not applicable; P_0, CH_4 production potential; R_{max}, the maximum CH_4 production rate; t_0, lag time; k, CH_4 production rate constant.

Table 4
Kinetic parameters of CH_4 production with respect to different FW/CM and S/I obtained from the modified Gompertz model and first-order model after long-term acclimatization.

Samples	FW/CM (VS/VS)	S/I (VS/VS)	Modified Gompertz model				First order model		
			P_0 (mL/g VS)	R_{max} (mL/day/g VS)	t_0 (d)	R^2	P_0 (mL/g VS)	k (day^{-1})	R^2
FW	/	0.57	750.6	237.7	0.18	0.9941	797.4	0.391	0.9911
CM	0	0.67	188.5	31.5	0.21	0.9871	219.3	0.179	0.9927
FW/CM	3.4	0.62	647.4	196.4	0.18	0.9925	690.7	0.372	0.9914
	2.5	0.62	609.7	185.0	0.14	0.9911	648.9	0.382	0.9942
	1.7	0.62	546.8	166.9	0.12	0.9909	580.5	0.390	0.9951
	0.8	0.62	447.0	144.6	0.04	0.9862	471.1	0.439	0.9986
	0.4	0.62	352.0	106.0	0.05	0.9864	372.4	0.404	0.9987
	0.3	0.62	296.8	80.4	0	0.9783	311.8	0.394	0.9992
	0.2	0.62	278.3	71.1	0	0.9766	291.5	0.381	0.9985
S/I	3.4	0.57	686.2	149.1	0	0.9689	725.7	0.313	0.9917
	3.4	0.68	643.4	109.2	0	0.9511	693.6	0.248	0.9894
	3.4	0.79	458.2	113.1	0	0.9689	492.7	0.325	0.9953
	3.4	0.91	147.8	13.4	0	0.8517	127.0	0.319	0.8580
	3.4	1.02	/	/	/	/	37.6	3.634	0.9116
	3.4	1.13	/	/	/	/	14.2	2617	0.2383

VS/VS, the cumulative biogas production at the S/I of 1.12 VS/VS was markedly decreased due to a large amount of VFA accumulation in the batch digester, as discussed in Section 3.1.4. Moreover, the maximum k value of 0.635 day^{-1} was achieved at the S/I of 0.07 VS/VS, which was consistent with the maximum P_0, as shown in Table 3. Therefore, the optimum S/I of approximately 0.07 VS/VS was used to start-up codigestion with FW and CM using the unacclimated sludge as reported in the authors' previous study (Xing et al., 2020b). As shown in Fig. 1d, the highest CH_4 yield of 696 mL/g VS was achieved at the S/I of 0.57 VS/VS using the acclimated sludge, which was consistent with the changes in P_0 and cumulative CH_4 production (Table 4). Meanwhile, the highest CH_4 yield was significantly higher than the previously reported CH_4 yield values in the range of 121.4–388 mL/g VS with similar feedstock, as shown in Table 6. For the seed sludge without acclimatization, the cumulative CH_4 yield visibly decreased as the S/I increased to 0.56 VS/VS (Fig. 1b). For the long-term acclimated sludge, however, the AD digester would not have inhibitory effects at the S/I of 0.57 VS/VS, and even at the S/I of 0.79 VS/VS (Fig. 1d). These results indicated that the long-term acclimatization of the codigestion with FW and CM also changed the optimum S/I. Thus, the FW/CM and S/I should both be further optimized when starting up a new codigestion using acclimatization sludge. Furthermore, the key start-up parameters, including the substrate mixture ratio and S/I, should be reoptimized to rapidly achieve stable and high conversion efficiency codigestion with different inoculum sources and feedstocks.

3.1.2. AD reaction ratios

As shown in Fig. 3, the four AD reaction ratios of codigestion with FW and CM at different FW/CM and S/I were presented with seed sludge without acclimatization and the acclimated sludge. For the seed sludge without acclimatization, the four conversion ratios for FW monodigestion were all higher than those for CM monodigestion (Fig. 3a), which was consistent with the k values, as shown in Table 3. Moreover, a lower hydrolysis conversion efficiency than the other three conversion ratios indicated that hydrolysis was the rate-limiting stage for the further biodegradability of FW and CM, which was in agreement with the results obtained by other studies (Gaur and Suthar, 2017; Zhai et al., 2015). As shown in Fig. 3a, the highest conversion efficiencies of hydrolysis, acidogenesis, acetogenesis, and methanogenesis were 83.6%, 89.2%, 89.2%, and 89.3%, respectively, at the optimum FW/CM of 2.5 VS/VS, which agreed with the maximum enhanced CH_4 yield percentage, as shown in Fig. 2a. For the long-term acclimated sludge, four similar reaction conversion efficiencies in the range of 80.7–87.3% were achieved at the S/I of 0.62 VS/VS and optimum FW/CM of 3.4 VS/VS (Fig. 3b). Furthermore, the hydrolysis reaction ratios of acclimated sludge were rapidly increased compared with that of seed sludge without acclimatization at the same FW/CM, indicating that hydrolytic enzymes with high activity and microorganisms with high hydrolysis

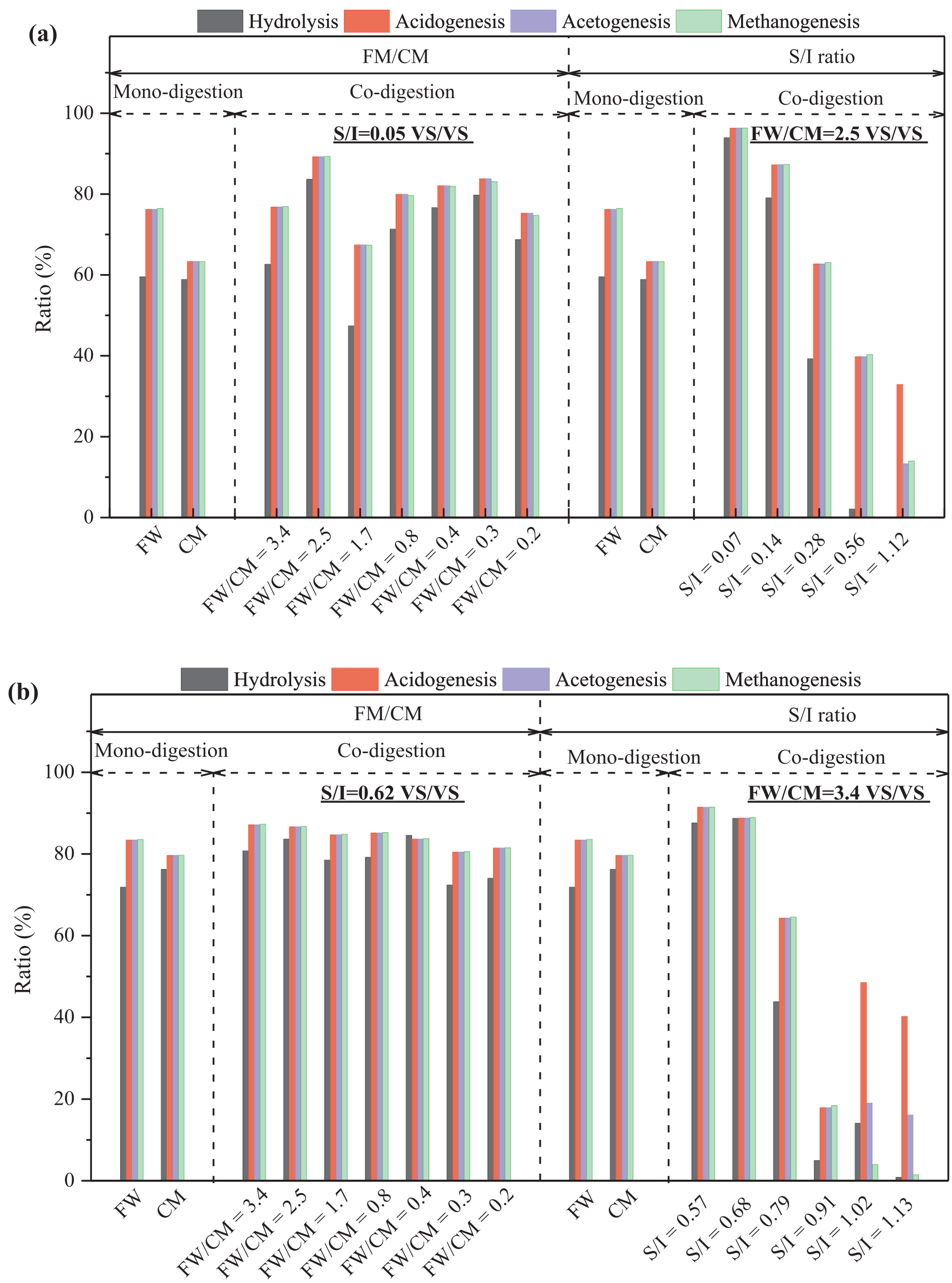

Fig. 3. Reaction ratios of the four AD steps of anaerobic codigestion with FW and CM values under different FW/CM and S/I (a) before and (b) after long-term acclimatization.

capacity were present in the codigestion sludge after long-term acclimatization as discussed in Section 3.2 and Section 3.3.

For the seed sludge without acclimatization, the maximum four reaction conversion efficiencies of more than 94% were reached at the S/I of 0.07 VS/VS and the optimum FW/CM of 2.5 VS/VS, as shown in Fig. 3a, which was consistent with the enhanced CH_4 yield percentage, as shown in Fig. 2a. As the S/I increased from 0.07 to 1.12 VS/VS, the four conversion efficiencies drastically decreased due to the high concentration of VFA accumulation and substrate overload, as reported by Guilford et al. (2019). The hydrolysis conversion efficiency was only 2.1% at the S/I of 0.56 VS/VS for the seed sludge without acclimatization (Fig. 3a). However, the hydrolysis conversion efficiency increased to 87.6% at a similar S/I of 0.57 VS/VS for the acclimated sludge (Fig. 3b). These results indicated that the four conversion rates of the acclimated sludge were significantly increased during long-term acclimatization through sequentially reduced HRTs, which was consistent with the authors' previous study that reported superior performance as the HRT decreased stepwise through long-term operation (Xing et al., 2020b).

3.1.3. Mass balances

Mass balance can be used to evaluate the CH_4 recovery potential in the AD system (Chen et al., 2019). The mass balance of anaerobic codigestion with FW and CM under different FW/CM and S/I before and after long-term acclimatization was calculated and is illustrated in Fig. 4. The particulate COD (PCOD) percentage of 81.9% in CM was higher than that in FW by approximately 23.6%. The PCOD percentages in the feedstock at the same S/I both increased when the FW/CM was

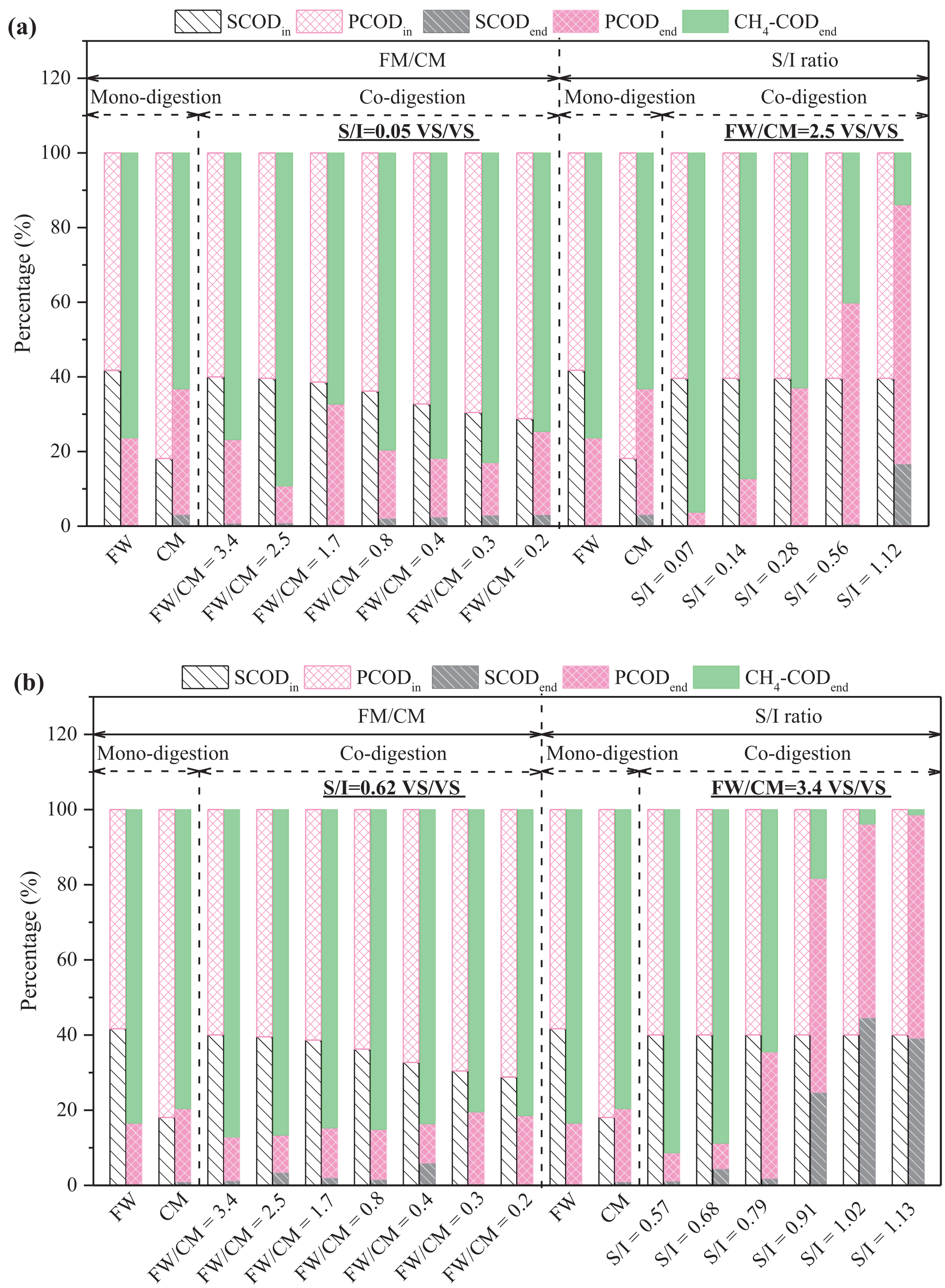

Fig. 4. Mass balances of anaerobic codigestion with FW and CM under different FW/CM and S/I before (a) and after (b) long-term acclimatization.

decreased from 3.4 to 0.2 VS/VS. For the seed sludge without acclimatization, the maximum CH_4-COD content of 89.3% was realized at the optimum FW/CM of 2.5 VS/VS and then changed from 2.5 to 3.4 VS/VS after long-term acclimatization. For the long-term acclimated sludge, the proportion of the PCOD also decreased from 22.4% to 11.5% at the optimum FW/CM of 3.4 VS/VS and a higher loading rate compared with the seed sludge. Furthermore, the end PCOD percentages ($PCOD_{end}$) in the monodigestion with FW and the monodigestion with CM both decreased from 23.6% to 16.4% and from 33.7% to 19.5%, respectively. These results indicated that the hydrolysis capacity of feedstock in the acclimated sludge was improved owing to the increased FW content in the feedstock and the changes in the microbial community, as discussed in Section 3.3.

As shown in Fig. 4, the CH_4-COD percentages both decreased when the S/I was increased at the corresponding optimum FW/CM in the codigestion system. Meanwhile, the proportions of PCOD also increased gradually, which was consistent with the changes in the CH_4 yield and hydrolysis reaction ratio, as shown in Figs. 2 and 3. As shown in Figs. 1 and 4, a high $PCOD_{end}$ of 59.2% was still maintained after approximately 25 days of codigestion at the S/I of 0.56 VS/VS using the seed sludge without acclimatization. However, the $PCOD_{end}$ percentage significantly decreased to 7.46% at a similar S/I of 0.57 VS/VS and a short digestion time of approximately 12 days using the acclimated sludge as the inoculum. Furthermore, a high CH_4-COD_{end} of 88.9% and low $PCOD_{end}$ of 6.77% could be continuously maintained as the S/I increased to 0.68 VS/VS under the optimum FW/CM of 3.4 VS/VS. Thus, the optimum S/I was increased to approximately 0.68 VS/VS after approximately 550 days of acclimatization, indicating that the digestion loading rate during the start-up period was significantly different using seed sludge with or without acclimation, much less with

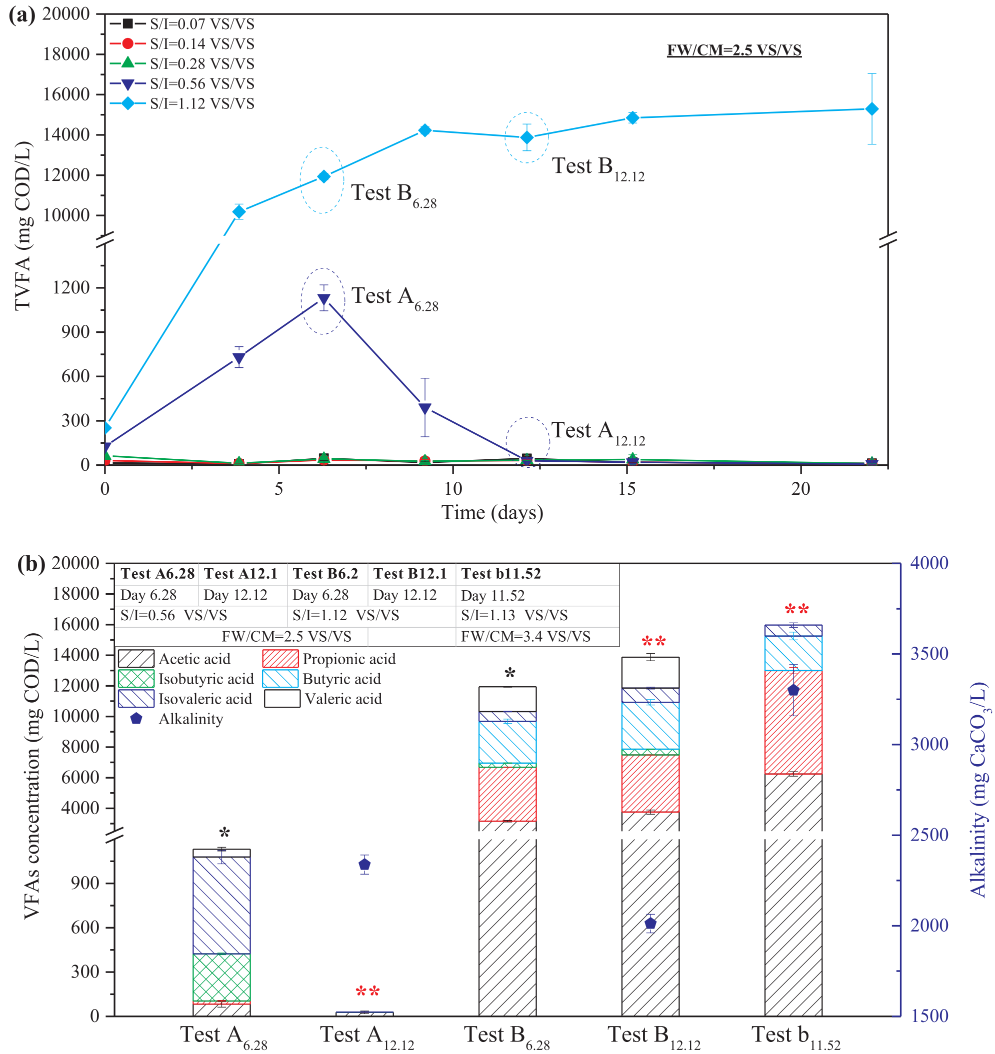

Fig. 5. VFAs under (a) different S/I using seed sludge and (b) various VFAs and alkalinity in the batch trails using the seed sludge (namely, Test A and Test B) and acclimated sludge (Test b).

different inoculum resources in practical engineering applications.

3.1.4. VFA accumulation and alkalinity

During the AD process, VFA accumulation can make a decrease in pH beyond the optimum pH ranges of methanogenic bacteria and then influence the digester performance and even cause failure (Zhou et al., 2011). The changes in VFAs under different S/I using seed sludge are presented in Fig. 5a. The TVFA concentration in the batch trial at an S/I of 0.56 VS/VS and optimum FW/CM of 2.5 VS/VS using the seed sludge (namely, Test A) reached the peak value of 1,132 ± 88 mg COD/L on Day 6.28 and then completely biodegraded to CH_4 on Day 12.12. Simultaneously, a high TVFA concentration of 11,937 ± 160 mg COD/L accumulated at an S/I of 1.12 VS/VS on Day 6.28 and then climbed to 13,876 ± 661 mg COD/L on Day 12.12. These results indicated that a high S/I beyond 1.12 VS/VS will lead to the destabilization of codigestion with FW and CM due to a large amount of VFA accumulation. Fig. 5b shows the various VFA concentrations and alkalinities in Test A and Test B with seed sludge and the batch trial at an S/I of 1.13 VS/VS on Day 11.52 with the acclimated sludge (namely, Test b). Similarly, a larger amount of VFA accumulated in Test b with acclimated sludge compared to Test B with seed sludge, as shown in Fig. 5b. Acetic acid, propionic acid, and butyric acid played dominant roles in the VFA accumulation at the end period of Test B and Test b. Meanwhile, the alkalinities in Test b and Test B were 3.30 and 2.01 g $CaCO_3$/L, respectively, suggesting that a higher alkalinity existed due to the increase in the optimum FW/CM. Reportedly, an alkalinity of 3.0–4.0 g $CaCO_3$/L was better, as it have better cushion against a decrease in pH resulting from an excessive increase in VFA (Wu et al., 2018). However, the pH at the end of Test b was low, with a value of 5.60 ± 0.07, and the alkalinity was 3.30 g $CaCO_3$/L. Meanwhile, the TVFA to alkalinity ratio was 4.84, which was beyond the reported threshold of 0.4–1.0 (Kafle and Kim, 2011, Li et al., 2017, Liu et al., 2012). Therefore, the suitable TVFA to alkalinity ratio was more suitable to use as an indicator for the stability of the codigestion during the high-rate operation period.

3.2. AD enzymes and lignocellulase

As shown in Table 5, the activities of AD enzymes and lignocellulase in the initial seed sludge and acclimated sludge were determined. During the AD process, hydrolysis is considered the rate-limiting step (Zhang et al., 2014). Proteins and polysaccharides in FW and CM were hydrolyzed into amino acids and glucose by protease and α-glucosidase (α-glu). Compared with those in the seed sludge, the activities of protease and α-glu in the acclimated sludge were increased 3.9 and 9.2 times, respectively, which was in accordance with the increasing hydrolysis reaction ratio of FW (CM) monodigestion (Fig. 3). For acidogenic enzymes, phosphotransacetylase (PTA) and acetate kinase (AK),

Table 5
Activity of AD enzymes and lignocellulase in the seed sludge and acclimated sludge after prolonged acclimation for more than 550 days under the initial optimal FW/CM of 2.5 VS/VS and OLR of 0.6–7.81 g VS/L/day.

Sample	AD enzymes (U/mL)										Lignocellulase (U/mL)									
	Hydrolytic		Acidogenic							Methanogenic	Ligninolytic			Hemicellulolytic				Cellulolytic		
	α-glu	Protease	PTA	AK	PTB	BK	Hase	CODH	CoA	F_{420}	Lip	Lac	MnP	xylanase	CMCase	xylitolase	xylan esterase	BG	EG	CBH
Seed sludge	0.03	0.37	0.13	0.05	0.09	0.06	0.05	0.13	0.08	0.71	0.09	0.08	0.07	0.125	352	1.24	0.001	0.02	332	0.01
Acclimated sludge	0.11	3.38	0.40	0.15	0.14	0.44	0.17	0.29	0.16	1.27	0.13	0.48	0.12	0.535	465	0.31	1.17	0.27	737	0.02

coA-transferase (CoA), phosphotransbutyrylase (PTB) and butyrate kinase (BK) are related to the production of acetic, propionic and butyric acids, respectively (Li et al., 2015). H_2 and CO_2 are effectively catalyzed by carbon monoxide dehydrogenase (CODH) and [FeFe] hydrogenase (Hase), which can be acetified via acetyl-CoA for the formation of acetic acid by homoacetogenic bacteria (Koo, 2020; Annie Modestra et al., 2020; Reginald et al., 2020). As shown in Table 5, the activities of these acidogenic enzymes increased 1.6- to 7.3-fold after long-term acclimatization, indicating that the capacity of biotransforming other micromolecule organics into acetic acid was improved in the acclimated sludge. The high CH_4 yield was also consistently correlated with coenzyme F_{420} (F_{420}) activity during anaerobic biodegradation (Fig. 2 and Table 5). Shamurad et al. (2020) reported that F_{420} content correlated statistically well with methanogenic activity, indicating that the measure of methanogenic populations and activities was reliable. These results indicated that the long-term acclimated sludge has high AD enzyme activities to improve biomass hydrolysis and mass transfer.

The AD system degrades lignocellulose via the synergistic action of various hydrolytic and oxidative enzymes. The lignocellulose-degrading enzymes consists of lignin-degrading enzymes (lignin peroxidase, Lip; laccase, Lac; and manganese peroxidase, MnP), hemicellulose-degrading enzymes (xylanase; carboxymethyl cellulose, CMCase; xylitolase; and xylan esterase), and cellulose-degrading enzymes (β-Glucosidase, BG; endoglucanase, EG; and cellobiohydrolase, CBH) (Xing et al., 2020b). These enzymes are grouped as Lac and heme-containing peroxidases (i.e., Lip and MnP), which are effective in treating industrial waste and organic waste through the biodegradation and decolorization process (Kumar and Chandra, 2020). Lignocellulose degradation can be effectively catalyzed by Lac. As shown in Table 5, the activity of Lac increased from 0.079 to 0.475 U/mL after long-term acclimatization, and this activity was higher than the activities of other ligninolytic enzymes. Hemicellulosic materials contain heterogeneous polysaccharides found in association with cellulose and lignin; however, the degradation of hemicelluloses requires different enzyme systems (i.e., xylanase, CMCase, xylitolase, xylan esterase) (Weiß et al., 2010; Vázquez et al., 2007). For cellulolytic enzymes, EG, CBH and BG represent the core set of enzymes for the hydrolysis of cellulose (Champreda et al., 2019). As shown in the authors' previous study (Xing et al., 2020b), the removal efficiencies of hemicellulose and cellulose were 58.8% and 78.3%, respectively, after long-term operation at high OLR, which was consistent with the high activity of lignocellulose-degrading enzymes, as shown in Table 5. Hemicellulose-degrading enzymes and cellulose-degrading enzymes can work together to degrade lignocellulose. As a result, the AD system was operated stably at a high OLR without significant inhibition, and the optimum S/I also improved, as mentioned above. Thus, high activity hydrolytic enzymes, including the AD enzyme and lignocellulose-degrading enzyme, were secreted in the codigestion after long-term acclimatization, which was the main reason for the changes in the optimum FW/CM for the feedstock and suitable S/I to start up a new codigestion system.

3.3. Microbial community analysis

The microbial communities in the initial seed sludge and acclimated sludge were analyzed as shown in Fig. 6. Only relative abundances (RAs) higher than 1% were shown in this study. As shown in Fig. 6a, *Pir3 lineage* (26.47%), *Anaerolineaceae* (12.53%), and *Christensenellaceae R-7* (12.2%) were the dominant bacterial genera in the seed sludge. In the acclimated sludge, *Prolixibacteraceae* (22.71%), *Syntrophomonas* (13.5%), *Candidatus Cloacimonas* (13.28%), and *DMER64* (12.02%) were selectively enriched compared to the seed sludge (Fig. 6a). The genera *Prolixibacteraceae* and *DMER64* belong to the phylum *Bacteroidetes*, which contains cellulose-degrading bacteria that produced cellulolytic enzymes in the AD system (Aryal et al., 2018). The representatives of the phylum *Bacteroidetes* are dominant bacteria after long-term acclimatization, which was consistent with the increasing

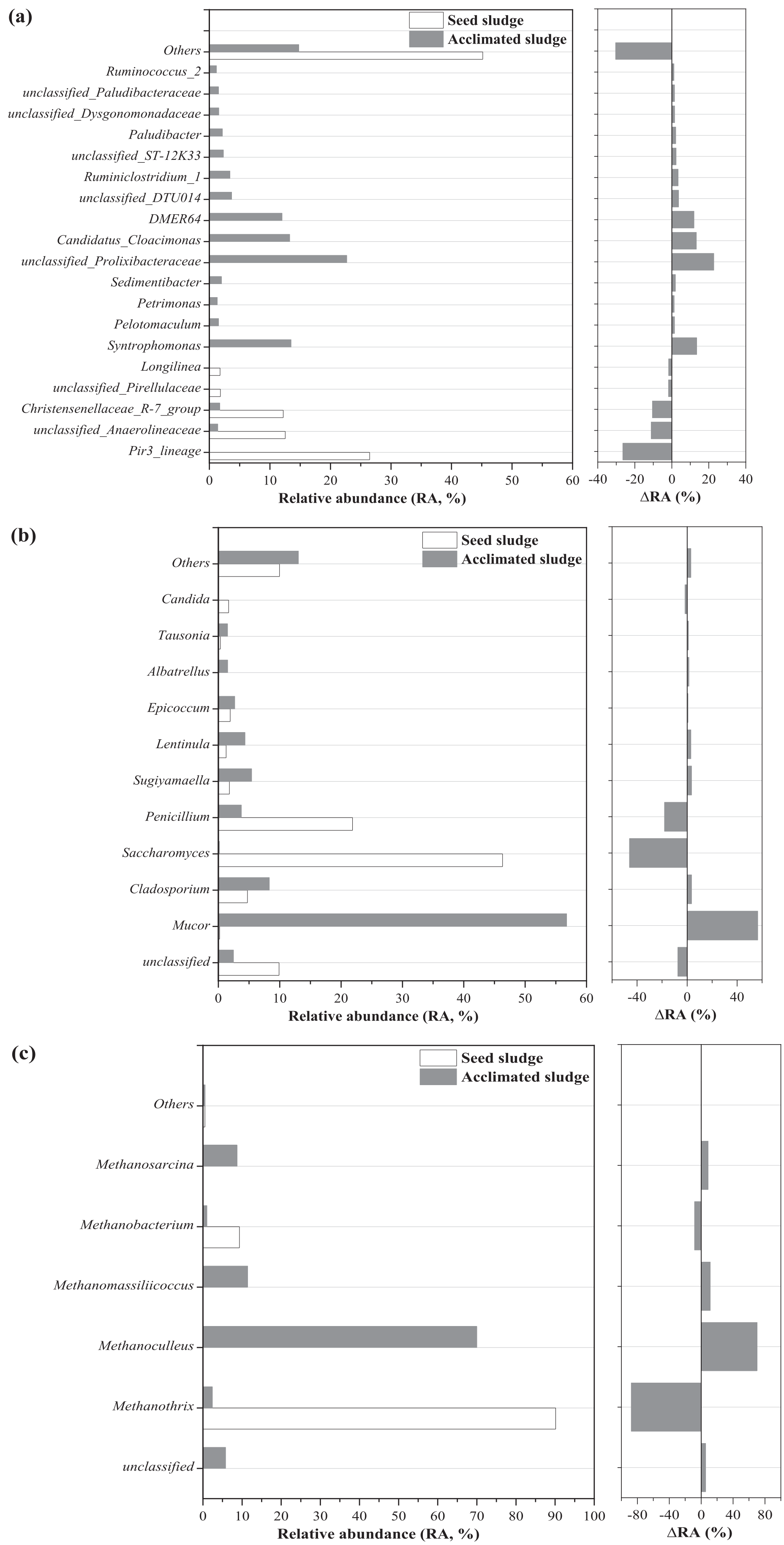

Fig. 6. Variation in the relative abundances (RAs) and RA increment (ΔRA) values of (a) bacteria, (b) fungi, and (c) archaea in seed sludge and acclimated sludge.

Table 6
Comparison of the optimum feeding mixture and S/I in anaerobic codigestion of FW and CM in this study and other studies.

Substrates	Inoculum source	Whether the inoculum was acclimatized with FW and CM	Type	Temp.(°C)	Substrate mixing ratio (VS/VS)	S/I (VS/VS)	CH_4 yield (mL/g VS)	References
FW + CM	Mesophilic anaerobic digested sludge from a normal operation anaerobic digester	No	Batch	35	FW/CM = 1.24	7:2 (v/v)	121.4–179.8	Zhai et al., (2015)
FW + CM	Laboratory-scale working digesters treating municipal solid waste.	No	Batch	35 ± 1	FW/CM = 0.92	less than1.44	311	El-Mashad and Zhang, (2010)
FW + CM	Anaerobically treated activated sludge	No	Batch	35 ± 1	FW/CM = 2	/	388	Zhang et al., (2013)
FW + CM	Swine waste treatment plant	No	Batch	35 ± 1	FW/CM = 1	0.94	298.6	Li et al., (2009)
FW + CM	Stable laboratory mesophilic anaerobic digester fed with CM and FW	Yes	CSTR	37	FW/CM≈0.79	/	250 ± 5	Neves et al., (2009)
FW + CM	Full-scale mesophilic anaerobic reactor at the brewery plant	No	Batch	39	FW/CM = 2.5	0.07–0.56	421–653	This study
FW + CM	Mesophilic anaerobic digester fed with FW and CM (FW/CM = 2.5 VS/VS) more than 550 days	Yes	Batch	39	FW/CM = 3.4	0.57–0.79	491–696	This study

secretion of cellulolytic enzymes for the transformation of cellulose to CH_4 (Table 5). The members of *Candidatus Cloacamonas* are hydrogen-producing syntrophs (Pelletier et al., 2008). In addition, members of *Syntrophomonas*, such as obligately anaerobic and syntrophic bacteria, oxidize VFAs; for example, butyrate into acetate (Zhang et al., 2017). With the increasing richness of *Syntrophomonas* and *Candidatus Cloacamonas*, VFA accumulation can be remitted at high OLRs (data not shown). The identified fungi in the acclimated sludge were mainly composed of five genera, as shown in Fig. 6b: *Mucor* (56.8%), *Cladosporium* (8.3%), *Sugiyamaella* (5.4%), *Lentinula* (4.4%), and *Penicillium* (3.7%). *Mucor* was the dominant fungal species and is responsible for secreting CMCase and cellulases for degrading crystalline cellulose (Baba et al., 2005). *Cladosporium* secretes Lac and EG, which are mainly xylan-degrading fungal strains (Gil-Durán et al., 2018). *Sugiyamaella* is a xylose-fermenting fungus that can secrete xylanolytic enzymes. *Lentinula* secretes hydrolytic (BG and laccase) and oxidative enzymes (MnP) to degrade lignocellulose (Chicatto et al., 2014). All of the above fungi secreted more lignocellulase to breakdown feedstock to organic acids compared to the seed sludge (Table 5), especially for the hydrolysis enzymes. These results indicated that the changes in the bacterial and fungal communities in the acclimated sludge improved the hydrolysis capacity of the FW and CM mixture and the contents of hydrolysis enzymes.

Fig. 6c shows the difference in the archaeal community in the seed sludge and acclimated sludge after long-term acclimatization. The most abundant archaea in both sludges were classified as acetotrophic methanogens (*Methanothrix*), hydrogenotrophic methanogens (*Methanoculleus*, *Methanomassiliicoccus*, and *Methanobacterium*), and mixotrophic microorganisms (*Methanosarcina*). As shown in Fig. 6c, *Methanothrix* (90.4%) played a major role in the seed sludge and then significantly decreased during the long-term operation as the OLR increased. Meanwhile, the RAs of hydrogenotrophic methanogens (*Methanoculleus* (70.0%) and *Methanomassiliicoccus* (11.5%)) were much higher than those of acetotrophic methanogens in the acclimated sludge. Compared to hydrogenotrophic methanogens, acetotrophic methanogens are more sensitive to OLR change (Westerholm et al., 2016). The doubling time of hydrogenotrophic methanogens was much shorter than that of acetotrophic methanogens (Bi et al., 2020). In addition, *Methanosarcina* (8.74%) is the most versatile methanogen that can biotransform different substrates and is more resistant to inhibitors (Capson-Tojo et al., 2018). Obviously, the predominant methanogenic archaea in the digester gradually changed from acetotrophic methanogens to hydrogenotrophic methanogens after long-term acclimatization, which had a close relationship with the F_{420} content in the digester sludge. Thus, the FW/CM should be optimized again after long-term acclimatization to reach higher conversion efficiency. Meanwhile, the S/I should be reoptimized when used as new inoculum sludge to start up another codigestion system.

Inoculum sludge plays a vital role in the feedstock degradation efficiency (Dechrugsa et al., 2013). As shown in Table 6, compared with previous studies, different FW/CM and S/I were adopted in the codigestion with FW and CM. The CH_4 yield of 491–696 mL/g VS was realized with a low FW/CM of 0.57–0.79 using the acclimated sludge in this study, which was still higher than those in other studies, even with high FW/CM in the range of 0.94–1.44 VS/VS (Table 6). Thus, different optimum feeding mixtures ratio and S/I were achieved even with a similar complex matter type, indicating that the makeup of the microbes and enzymes in the inoculum sludge were the main reasons for the changes in the optimum feeding mixture ratio and S/I. Furthermore, to further improve the AD process efficiency and system stability, the optimization of start-up parameters involved in the codigestion system in engineering was necessary even with a similar feedstock type. Moreover, the optimal FW/CM and S/I should also be reoptimized after long-term acclimatization due to changes in the microbial community and the secreted AD enzyme contents in the acclimated sludge.

4. Conclusions

This study demonstrated that a long-term acclimatization of more than 550 days changed the optimum FW/CM and S/I in anaerobic co-digestion with FW and CM. For the seed sludge without acclimatization, the optimal FW/CM and S/I were 2.5 VS/VS and less than 0.07 VS/VS, respectively. After long-term acclimatization, the optimum FW/CM and S/I for the acclimated sludge were 3.5 VS/VS and less than 0.57 VS/VS, respectively. Changes in enzyme activities and the microbial community structure during the long-term acclimatization process were the main reasons for the changes in the optimum FW/CM and S/I.

CRediT authorship contribution statement

Bao-Shan Xing: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Yule Han:** Conceptualization, Methodology, Investigation. **Sifan Cao:** Investigation. **Xiaochang C. Wang:** Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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