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High-rate mesophilic co-digestion with food waste and waste activated sludge through a low-magnitude increasing loading regime: Performance and microorganism characteristics

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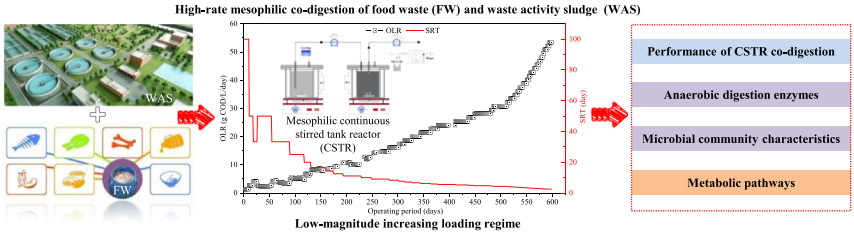
HIGHLIGHTS

- A low-magnitude loading regime can be used to realize high-rate co-digestion.
- Stable and high-rate CSTR co-digestion can be performed at SRT of 2.56–2.63 days.
- A maximum CH_4 production of 12.9 L/L/day was achieved in mesophilic CSTR digestion.
- The high RA of *Methanosarcina* was the main reason for the high production of CH_4.
- The main metabolic pathway in the high-rate mesophilic CSTR digestion was analyzed.

GRAPHICAL ABSTRACT



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ABSTRACT

To achieve a high-rate operation of co-digestion with waste activated sludge (WAS) and food waste (FW) for biogas production, a low-magnitude loading regime was investigated in a mesophilic continuous stirred tank reactor (CSTR) over long-term operation for approximately 600 days. The results showed that high-rate mesophilic CSTR co-digestion was realized using the low-magnitude loading regime. A maximum methane production of 12.9 L/L/day was achieved in the mesophilic CSTR co-digestion at an organic loading rate (OLR) of 48.1 g-COD/L/day. Moreover, high-efficiency and stable mesophilic CSTR co-digestion can still be performed at OLR of 50.8–52.1 g-COD/L/day and solid retention time (SRT) of 2.56–2.63 days without volatile fatty acid (VFA) accumulation. A high methane yield, hydrolysis conversion ratio, and methanogenic activity and the key anaerobic digestion enzymes were all maintained during the high-rate operation period. 16S rRNA gene sequencing results indicated that the relative abundance of the class *Clostridia* and genus *Methanosarcina* could reach 85.0% and 97.3%, respectively, corresponding to a high hydrolysis rate and VFA conversion rate. The metabolic capability of the genus *Methanosarcina* was the main reason for the highly efficient and stable operation of the mesophilic CSTR co-digestion. Using metagenomic analysis, *Methanosarcina barkeri* and *Methanosarcina flavescens* were established as the main methane-producing species during high-rate mesophilic CSTR co-digestion. The enrichment of the genus *Methanosarcina* through a low-magnitude loading regime is a promising method for realizing the highly efficient and stable operation of co-digestion with WAS and FW for biogas production at low retention times.

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1. Introduction

Waste activated sludge (WAS) and food waste (FW) are becoming public concerns and global environmental challenges due to rapid urbanization worldwide. Anaerobic digestion (AD) is a biological process that converts complex organic wastes into renewable biogas. Compared to mono-digestion, anaerobic co-digestion of WAS and FW is an eco-friendly option to overcome the disadvantages of mono-digestion systems (Bartocci et al., 2020; Hagos et al., 2017; Li et al., 2020b). Current wastewater treatment plants (WWTPs) can be turned into energy producers through anaerobic co-digestion of WAS and FW (Yin et al., 2016). Despite the significant amount of attention devoted to the co-digestion of WAS and FW in recent years, challenges remain to be overcome. Studies show that anaerobic co-digestion typically has a long retention time (HRT) and low organic loading rate (OLR) (Hagos et al., 2017; Iacovidou et al., 2012). Therefore, achieving stable and high efficiency co-digestion under low HRTs can further decrease the volume of digesters and plant footprints in practical applications.

The biogas production efficiency of the co-digestion process is affected by different operating parameters (Li et al., 2017; Wang et al., 2020). Most importantly, microbial communities play an essential role in the AD process. The AD system is often problematic due to the slow growth and sensitivity of the methane-producing archaea under the environmental conditions. To avoid the overloading and washing out of methanogens, AD in continuous stirred tank reactors (CSTRs) commonly operates at OLRs below the optimum capacity and solid retention times (SRTs) on the order of twenty days or more (Appels et al., 2008). Mesophilic digestion systems can treat effectively up to an OLR of 18.5 g-VS/L/day (Dai et al., 2013), whereas 30.2 g-VS/L/day can be treated in a thermophilic digester (Li et al., 2017). By comparison to other methanogens, it has been reported that *Methanosarcina* sp. have high growth rates with doubling times in the range of 1.0–1.2 days and are tolerant to NH_4^+-N, salt, and acetate concentrations up to 7000 mg/L, 18 g Na^+/L, and 15 g-COD/L, respectively (De Vrieze et al., 2012). Thus, it can be stated that *Methanosarcina* sp. are able to realize stable methanogenesis at high OLRs and low SRTs. Furthermore, the single-stage CSTR digestion will be more economical in view of operating cost. However, to our knowledge, no other studies have reported the selective enrichment of a *Methanosarcina* sp. in single-stage CSTR co-digestion with WAS and FW to date.

Microbial community composition in the co-digestion process shifts with several operative conditions. To successfully develop a high-rate and stable co-digestion technology for WAS and FW treatment, a few important strategic methods have been developed to resolve process failures (Mehariya et al., 2018). Moreover, several engineering and biochemical methods have been examined, including pretreatment approaches, altered reactor designs, and bioaugmentation. The magnitude of changing operational parameters could transform and change the microbial community in the bioprocess treatment system (Li et al., 2020a). In a previous study, mesophilic CSTR co-digestion of WAS and FW failed under an OLR of 16.2 g-VS/L/day after approximately 180 days of operation in a high-frequency feeding digester (Li et al., 2017). High-magnitude loading regimes have been applied in a mesophilic CSTR, and the OLR changed from 11.1 to 16.2 g-VS/L/day, which led to a large substrate shock and then led to an imbalance between acidification and methanation. To date, low-magnitude loading regimes in AD systems to achieve stable and high-rate digestion have not been investigated.

To fill in the gap in state of the art AD systems, the aim of this paper is to assess the feasibility of low-magnitude loading regimes to achieve high-rate mesophilic co-digestion of WAS and FW in a CSTR digester operated at sequentially reduced SRTs. Then, we studied the dynamic behavior of microorganisms during long-term operation for 600 days. Furthermore, the main metabolic pathways in the high-rate mesophilic CSTR co-digestion with WAS and FW were also analyzed from the view of AD enzymes and microorganisms through metagenomics sequencing technology.

2. Materials and methods

2.1. Feedstock and seed sludge

The WAS collected from a WWTP in Xi'an, China. The FW was prepared based on the characteristics of FW in China as we have provided in the earlier study (Li et al., 2017). To obtain higher methane production, the feedstock is defined as the mixture of FW and WAS at a ratio of 4:1 based on wet mass (Dai et al., 2013). The FW and WAS mixture was then crushed for 10 min using a blender. The total solid (TS) concentrations of feedstock were maintained as a TS content of approximately 9.5% with the dilution of tap water and stored at 4 °C by a cooler. The seed sludge was taken from a full-scale mesophilic anaerobic reactor of a brewery plant in Xi'an, China. The characteristics of the feedstock and seed sludge used in this study are summarized in Table 1.

2.2. Reactor configuration and their operation

The whole experiment was conducted via a lab-scale CSTR with working volume as 0.7 L. The temperature of the reactor was maintained under mesophilic conditions (39 ± 1 °C) by a heater and a water jacket. Feedstock was semi-continuously pumped to the CSTR by a peristaltic pump from the substrate tank. During the start-up period, the reactor was initially inoculated with 0.7 L of the seed sludge and fed at a low OLR of 1.40 g-COD/L/day. Subsequently, the OLRs of the CSTR were increased by filling and drawing over a gradually shortened SRT. In each operation cycle, the digestion was extracted at the rate of 7 mL/10 s for once, and then the same volume of feedstock was feed to the CSTR. The feeding time interval was the same under a constant OLR. With increasing frequency of pump operation, the feeding time interval was reduced gradually. Meanwhile, the feeding rate under different SRTs during the long-term operation of the CSTR system is shown in Fig. 1a. During the whole period, the reduction extent of SRT (ΔSRT) exponentially decreased from 50 to 0.064 days as the OLR increased gradually from 1.40 to 53.5 g-COD/L/day (Fig. S1).

2.3. Specific methanogenic activity (SMA) tests

To determine the methane production rate of co-digestion sludge using either individual volatile fatty acids (VFAs) or H_2 combined with CO_2 as substrates, SMA tests were performed in 120-mL serum bottles. Either sodium acetate, sodium propionate, sodium butyrate, or sodium valerate were used as the substrate at the initial concentration

Table 1
Physicochemical properties of seed sludge and feedstock.

Parameter	Feedstock	Inoculum
TS (g/L)	84.2 ± 9.5	29.6
VS (g/L)	72.6 ± 9.2	12.4
TCOD (g/L)	114 ± 1.3	16.7
SCOD (g/L)	47 ± 0.14	0.81
pH	4.23 ± 0.95	7.55
Protein (g/L)	4.1 ± 0.05	0.02
Carbohydrate (g/L)	2.53 ± 0.02	0.32
NH_4^+-N (mg/L)	712 ± 46	102
Alkalinity (g $CaCO_3$/L)	0.00	16.5
Acetic acid (mg/L)	0.97	8.15
Propionic acid (mg/L)	0.05	0.00
Butyric acid (mg/L)	0.00	0.00
Valeric acid (mg/L)	0.00	0.00
Isovaleric acid (mg/L)	0.00	2.90
C (%)	41.9 ± 0.14	/
H (%)	5.29 ± 0.07	/
O (%)	29.4 ± 1.77	/
N (%)	5.53 ± 0.02	/
S (%)	0.92 ± 0.01	/

Notes: "/" means not applicable.

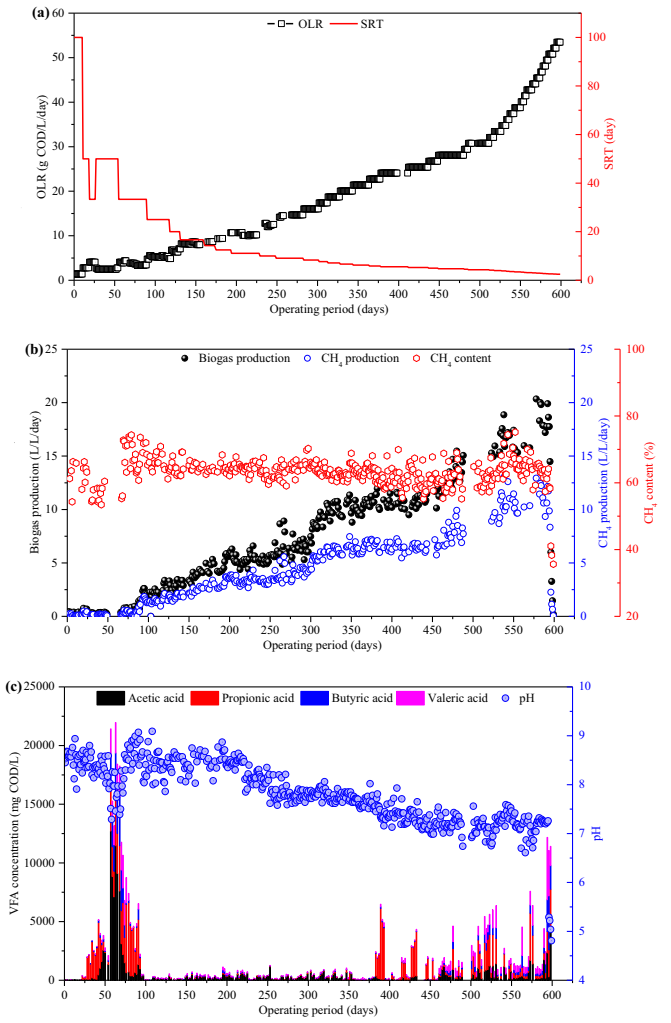

Fig. 1. Variation in the (a) OLR and SRT; (b) biogas production, methane production, and methane content; (c) VFA concentration and pH temporal profiles during the whole experiment of the CSTR.

of 5 g-COD/L each. 30 mL of codigested sludge was added as inoculum, which was directly taken from the CSTR under SRTs of 9.09, 4.76, 3.13, and 2.56 days before use, without any pretreatment. The headspace in serum bottles were immediately purged with N_2 for 2 min to ensure anaerobic conditions and then sealed with a rubber septum. The serum bottles were then placed into a shaking water bath at 39 ± 1 °C and agitated at 120 rpm. The SMA was determined using data for the fastest methane production, which simulated using the modified Gompertz model as described in an earlier study (Li et al., 2017).

2.4. Microbial community analysis

Samples were collected from the inoculum and CSTR on Day 411 and Day 594, and the diversity of the microbial communities was characterized by using high-throughput sequencing technology. To further characterize the predominant species of microbial communities, samples were also collected from the mixing effluent digestate from Day 501 to Day 545. DNA was extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA), according to the manufacturer's instructions. A polymerase chain reaction (PCR) targeting 16S rRNA genes was performed using two primer pairs, i.e., 341F/805R for bacteria (5′-CCTACGGGNGGCWGCAG-3′)/(5′-GACTACHVGGGTATCTA ATCC-3′) and 349F/806R for archaea (5′-GYGCASCAGKCGMGAAW-3′)/(5′-GG ACTACVSGGGTATCTAAT-3′). After purification and quantification, the PCR products of the V3-V4 region of the 16S rRNA gene were sequenced using an Illumina HiSeq platform at Sangon Biotech Shanghai Co., Ltd., China. Meanwhile, the metagenomic sequencing analyses were performed on an Illumina Genome Analyzer (HiSeq X-ten, Illumina Inc., San Diego, CA, USA) at Sangon Biotech Shanghai Co., Ltd., China.

2.5. Physico-chemical analytical methods

The soluble COD (SCOD), total COD (TCOD), TS, VS, alkalinity, and NH_4^+-N were analyzed according to the standard methods (APHA, 2005). Soluble proteins and carbohydrates were determined according to the Lowry-Folin method and the anthrone-sulfuric acid method, respectively (Herbert et al., 1971; Lowry et al., 1951). The pH of the digestate and substrate was measured by a pH meter (Horiba, Kyoto, Japan). The volumetric biogas production was measured daily by a wet gas mater. The composition of the biogas and VFAs were measured as described by Li et al. (2017). The free ammonia (FA) concentration in the digestate was calculated according to the method proposed by Anthonisen et al. (1976). The contents of AD enzymes analysis were carried out in accordance with the authors' previous study (Xing et al., 2020).

3. Results and discussion

3.1. Performance of CSTR with OLR increasing

The co-digestion of WAS and FW in a single-stage mesophilic CSTR for biogas production is mostly used in engineering applications. The low-magnitude OLR increasing method was used to achieve stable and high-rate co-digestion with WAS and FW. As shown in Fig. 1, changes in biogas production, methane production, VFA production, and pH occurred with the increase in low-magnitude OLRs in the mesophilic CSTR. The maximum methane production of 12.9 L/L/day was realized with an OLR of 48.1 g-COD/L/day and an SRT of 2.78 days. Meanwhile, the TVFA concentrations were in the range of 0.98 to 1.16 g-COD/L, and the pH in the effluent digestate was 7.2 ± 0.1 from Day 578 to Day 581. According to a previous study, a neutral pH of 6.8–7.5 is the most favorable for methanogen metabolism, and AD processes are generally reported to be vulnerable at TVFA of 3.5 g-COD/L or greater (Mehariya et al., 2018; Wang et al., 2009). Therefore, high-rate co-digestion of WAS and FW can stabilize operation of the mesophilic CSTR through long-term acclimatization using the low-magnitude OLR increasing method.

Compared with the previous study (Li et al., 2018), a maximum methane production of 3.72 ± 0.18 L/L/day was achieved in the mesophilic CSTR with the same feedstock but readily broken down under OLRs and SRTs of 25.1 ± 1.8 g-COD/L/day and 5 days, respectively. High-magnitude increases in OLR from 17.0 ± 0.8 to 25.1 ± 1.8 g-COD/L/day cause a large loading shock for microorganisms, even when adopting the high-frequency feeding strategy (Li et al., 2017). On the other hand, a low methane production of 10.3 ± 0.9 L/L/day was achieved in a thermophilic CSTR under a similar OLR of 48.0 ± 1.7 g-COD/L/day. Meanwhile, the TVFA concentration in the thermophilic CSTR was 4.07 ± 1.48 g-COD/L, a value higher than that of the mesophilic CSTR in this research. Therefore, the digestion treatment capacity of the mesophilic CSTR through the low-magnitude OLR increasing method can rival that of the thermophilic CSTR using the high-magnitude OLR increasing method. However, the operation of a high-rate thermophilic digester is harder to control and requires more energy to maintain the digester temperature (Hagos et al., 2017), indicating that more bioenergy is recovered using the low-magnitude OLR increasing strategy in the mesophilic CSTR. A lower energy supply is more suitable to reduce the carbon footprint and to transform a WWTP from an energy consumer to an energy exporter.

During the start-up period (1–117 days), the TVFA concentrations gradually increased to 22.0 g-COD/L and then decreased to 0.13 g-COD/L

under an SRT of 25 days to adapt to the new environment (Fig. 1c). Moreover, the TVFA concentrations in the effluent digestate were in the range of 11.1–12.2 g-COD/L under an SRT of 2.5 days during the end period (595–599 days). The lower TVFA concentration with a ten-fold loading rate was mainly due to the growth of more robust microorganisms, as shown in Section 3.3. Based on the above results, using different reactor configurations, feedstock styles, and thermophilic AD processes may achieve a higher loading rate operation by using the low-magnitude loading method, but this hypothesis needs further investigation in future studies.

3.2. Buffering capacity and degradation efficiency

3.2.1. VFA/alkalinity and FA

The buffering capacity of the co-digestion process can be evaluated through the alkalinity and NH_4^+-N concentration (Li et al., 2017; Qiao et al., 2013). As shown in Fig. 2, as the OLR increased to 53.7 g-COD/L/day, the alkalinity and NH_4^+-N concentrations in the mesophilic CSTR fluctuated in the ranges of 4.1–7.8 g $CaCO_3$/L and 756–1748 mg/L, respectively. On the one hand, the TVFA to alkalinity (TVFA/Alkalinity) ratio, which is an indicator of AD system stability, was linearly positively correlated with the TVFA concentration ($R^2 = 0.9699$). According to previous studies, a TVFA/Alkalinity ratio of 0.4–1.0 is considered to be the threshold for digester stability (Kafle and Kim, 2011; Li et al., 2017; Liu et al., 2012). These results indicate that TVFA was the main factor influencing the co-digestion stability in this study. Thus, the conversion rate of VFAs is the key factor for stable operation under high loading conditions, which can be realized through the high activity of AD enzymes and a sufficient amount of methanogenic biomass. As shown in Figs. 1 and 2a, a stable performance could still be realized at an OLR of 50.8 g-COD/L/day and SRT of 2.63 days from Day 585 to Day 590, and the corresponding TVFA/Alkalinity ratio of 1.26 is beyond the reported threshold values. Furthermore, severe deterioration also occurred with a TVFA/Alkalinity ratio of 2.04 at an OLR of 53.5 g-COD/L/day and SRT of 2.5 days during the end period. On the other hand, NH_4^+-N can combine with CO_2 to form alkali chemicals that could improve the buffering capacity (Qiao et al., 2013). As reviewed by De Vrieze et al. (2012), *Methanosarcina* sp., which is one of the most dominant methanogenic communities, as shown in Section 3.3, are reported to be tolerant to NH_4^+-N up to 7000 mg/L. However, at a level of 250 mg NH_3/L, FA exhibits severe inhibitory effects on methanogenic activity (Yenigun and Demirel, 2013). As shown in Fig. 2b, FA concentrations of 0.5–120 mg NH_3/L were achieved as the OLR increased to 53.5 g-COD/L/day and the SRT decreased to 2.5 days, indicating that NH_4^+-N toxicity and FA inhibition were not the limiting factors to achieve the high degradation capacity of mesophilic CSTR co-digestion with WAS and FW.

3.2.2. Methane yield, TCOD removal efficiency, and mass balance

A theoretical methane yield of 312 mL/g-COD was calculated according to the elemental composition of the feedstock (Table 1) and the Buswell equation (Buswell and Mueller, 1952). As shown in Fig. 3a, the variation in methane yield was presented under different OLRs. During the start-up period (1–117 days), the methane yield gradually increased to 270 mL/g-COD as the OLR increased to 5.11 g-COD/L/day. During the following operation process, an average methane yield of 288 ± 38 mL/g-COD was realized, and the OLR gradually increased from 5.11 to 52.1 g-COD/L/day to a low extent (Figs. 3a and S1). The methane yield in the mesophilic AD process was larger than that of the thermophilic co-digestion process (approximately 214 ± 19 mL/g-COD) with the same feedstock and reactor configuration under a high loading rate of 48 g-COD/L/day compared with that reported by Li et al. (2017). The reason for this is mainly due to the high SMA achieved in the present study, as discussed in Section 3.2.3, which is also consistent with the low TVFA concentration in the mesophilic CSTR, as shown in Fig. 1. Moreover, the methane yield in the mesophilic CSTR using the low-magnitude OLR increasing strategy was higher than 184–219 mL/g-COD with the high-magnitude OLR increasing strategy under OLRs in the range of 6.27 to 17 g-COD/L/day (Li et al., 2017), which was consistent with the high hydrolysis conversion ratio of 64.0–79.9%

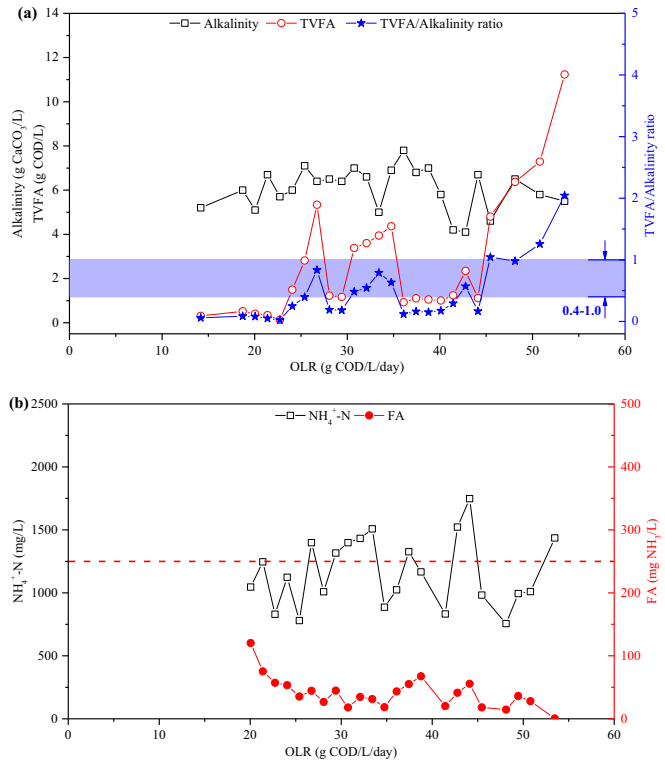

Fig. 2. Changes in (a) the alkalinity, TVFA, and TVFA/Alkalinity ratio; and (b) NH_4^+-N and FA in the mesophilic CSTR co-digestion with WAS and FW.

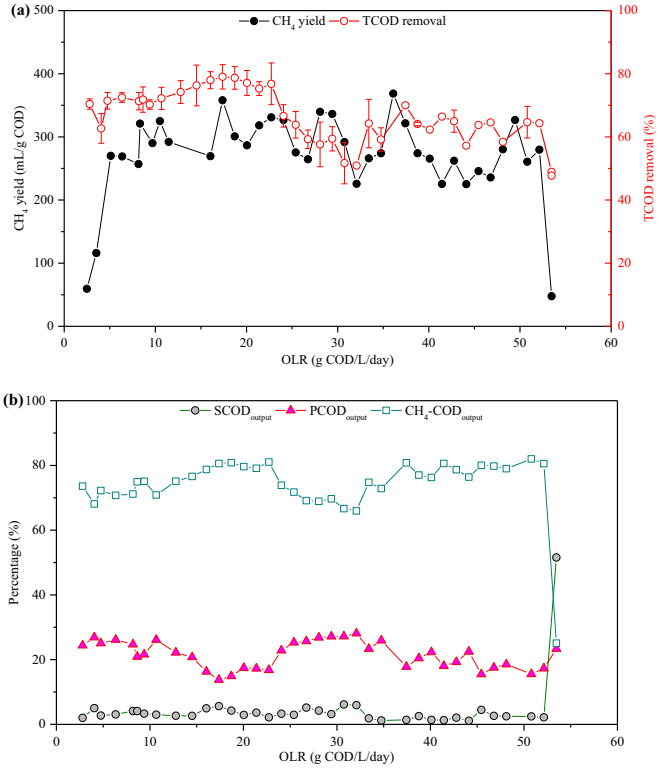

Fig. 3. Variation in (a) CH_4 yield and TCOD removal and (b) mass balances under different OLRs.

compared with the authors' previous study (30.3–32.1%). As a result, efficient and stable co-digestion with high hydrolysis and high SMA can be achieved in the mesophilic CSTR using the low-magnitude loading increasing method.

The TCOD removal efficiency in the mesophilic CSTR under different OLRs is presented in Fig. 3a. After the start-up period (1–117 days), the TCOD removal efficiency gradually increased from 71.4 ± 2.6% to 79.0 ± 3.9% as the OLR increased from 5.11 to 18.7 g-COD/L/day from Day 118 to Day 327, and the corresponding SRTs concomitantly decreased from 25 to 7.14 days. As a crucial operational parameter, SRT plays an important role in keeping the functional microbial groups in balance, and 12–30 days is required to co-digestion WAS with FW (Dai et al., 2013; Wang et al., 2020). Thus, the TCOD removal efficiency was further decreased to 50.9%, the SRT decreased to 4 days and the OLR increased to 33.4 g-COD/L/day from Day 328 to Day 526 (Fig. 3a). However, the TCOD removal efficiency increased to 70.0% under an SRT of 3.57 days and then fluctuated between 57.2% and 66.4% under OLRs of 38.7–50.8 g-COD/L/day and SRTs of 3.45–2.62 days. These results indicate that a robust microbial community was gradually constructed with sequentially reduced SRTs, as discussed in Section 3.3.

The mass balance for the mesophilic CSTR during the steady period under different OLRs is shown in Fig. 3b. A low average SCOD percentage of 3.2 ± 1.4% and a high average CH_4-COD percentage of 75.4 ± 4.6% were achieved with the OLRs increasing from 1.40 to 52.1 g-COD/L/day and the SRTs decreasing from 100 to 2.56 days throughout the experimental period, except for Day 595 to Day 599. After the day of 520 (i.e., OLR > 33.4 g-COD/L/day), the duration under different OLRs was in the range of 1.11 to 2.80 SRTs as shown in Fig. S1. Meanwhile, the ΔSRT was further decreased from 0.166 to 0.064 day making the corresponding SRT changed slowly from 4.0 to 2.5 days through 80 days operation. As shown in Figs. 1 and S1, the CSTR co-digestion was operated more than 3 SRTs at OLR of 50.8–52.1 g-COD/L/day and SRT of 2.56–2.63 days. As shown in Fig. 3, high methane yield of 261–280 mL/g-COD and CH_4-COD_{output} percentage of 80.5–82.0% were stable realized at SRT of 2.56–2.63 days. These results suggested that the balance between acidification and methanation can still be maintained in the mesophilic CSTR even at SRT of 2.56–2.63 days in this study through the low-magnitude loading increasing regime. Lower SRT/HRT values in CSTRs means more waste is treated, more biofuel is generated, and more time is saved while utilizing the same facility (Nges and Liu, 2010). As shown in Fig. 1c, a large amount of TVFA (approximately 11.2 g-COD/L) accumulated at an SRT of 2.5 days and an OLR of 53.5 g-COD/L/day, which made the pH rapidly drop to 4.81. Then, the low pH conditions inhibited the activity of methanogens (Sun et al., 2020), which was consistent with the low methane yield of 47.7 mL/g-COD, TCOD removal efficiency of 47.7%, CH_4-COD percentage of 25.1%, and high SCOD percentage of 51.6% from Day 595 to Day 599 (Fig. 3). Thus, a threshold SRT of 2.5 days for AD process balance was found in the mesophilic CSTR co-digestion with WAS and FW in this study. On the other hand, the change in the particulate COD (PCOD) percentage was negatively correlated with that in the TCOD removal efficiency and CH_4-COD percentage, as shown in Fig. 3. Moreover, a low PCOD percentage in the digester means a high hydrolysis rate, which has a close relationship with the bacterial communities and the secreted hydrolysis enzyme activity. Thus, a high rate of hydrolysis and methanogenesis were the two key points for the high degradation efficiency and stable operation of the high-rate mesophilic CSTR co-digestion.

3.2.3. Conversion ratio, SMA, and AD enzymes

As shown in Fig. 4, the hydrolysis, acidogenesis, acetogenesis, methanogenesis conversion ratios, and SMA under different OLRs were determined, and the ten key AD enzymes in the mesophilic CSTR were measured during the end period. Throughout the experimental period, the conversion ratio of hydrolysis was lower than that of acidogenesis, acetogenesis, and methanogenesis (Fig. 4a). Furthermore,

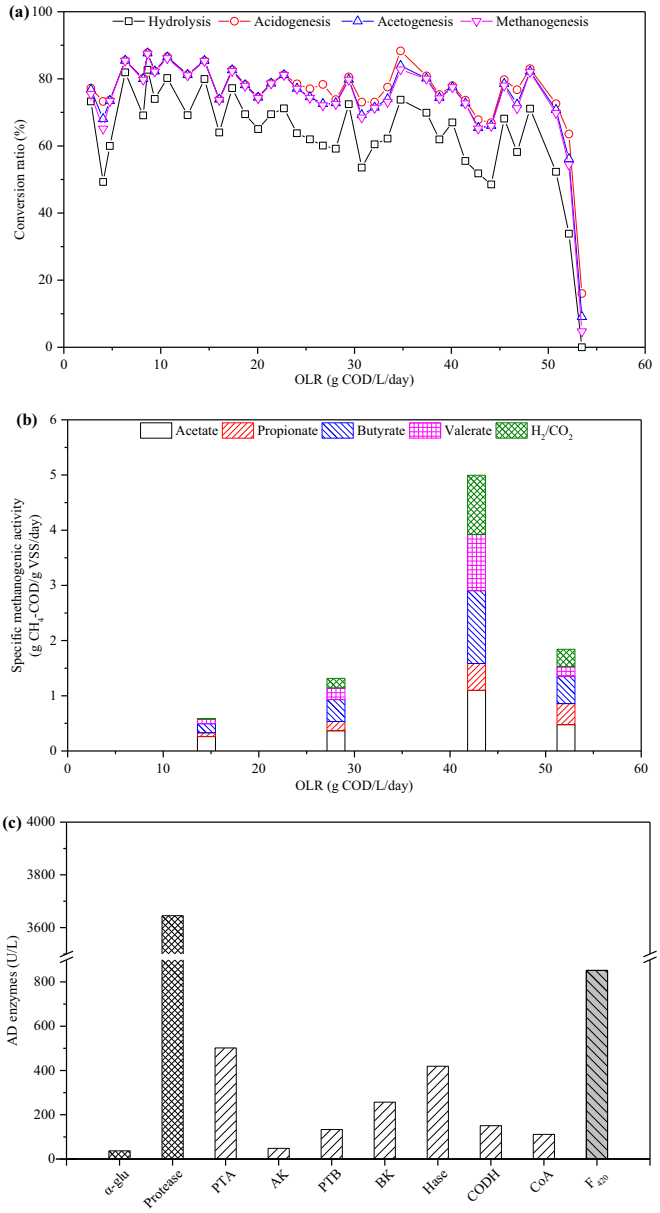

Fig. 4. Hydrolysis, acidogenesis, acetogenesis, and methanogenesis conversion ratios (a) and SMA (b) under different OLRs; hydrolytic (α-glu, Protease), acidogenic (PTA, AK, PTB, BK, Hase, CODH, and CoA), and coenzyme F_{420} levels (c) in the mesophilic CSTR during the end period.

the former was linearly positively correlated with the latter (R^2 = 0.8817, 0.9275, and 0.9246, respectively), and the conversion ratios of acidogenesis, acetogenesis, and methanogenesis were synchronous and similar. These results suggested that hydrolysis was the rate-limiting step for mesophilic co-digestion with WAS and FW, which is consistent with previous studies (Carrère et al., 2010; Lee et al., 2019). As shown in Fig. 4a, a high hydrolysis of 52.3% and methanogenesis of 69.8% were still maintained in the mesophilic CSTR as the OLR increased to 50.8 g-COD/L/day and SRT reduced to 2.63 days. However, a low hydrolysis of 20.2% and methanogenesis of 43.4% were observed in the thermophilic CSTR under a lower OLR of 48.0 g-COD/L/day and a longer SRT of 3 days by feeding the same feedstock using the larger-magnitude loading increasing regime, as reported by Li et al. (2017). As shown in Figs. 1 and 4a, a large amount of VFA accumulation and a sharp reduction in hydrolysis and methanogenesis conversion ratios occurred as the OLR further increased from 50.8 to 53.5 g-COD/L/day, which may be due to the imbalance between the proliferation and washout of AD

microorganisms in the mesophilic CSTR at a low SRT of approximately 2.5 days.

As shown in Fig. 4b, the SMA values of individual VFAs and H_2/CO_2 in the mesophilic CSTR first increased as the OLR increased and then decreased as the OLR further increased to 53.5 g-COD/L/day. Compared with the SMA values in the co-digestion sludge at an OLR of 28.1 g-COD/L/day, however, higher SMA values at an OLR of 52.1 g-COD/L/day were achieved. Moreover, the SMA values of sodium acetate, sodium propionate, and sodium butyrate of 0.48, 0.38, and 0.49 g CH_4-COD/g-VS/day at OLR of 52.1 g-COD/L/day (SRT of 2.56 days) were higher than those of the mesophilic CSTR under an SRT of 7.5 days (i.e., 0.203, 0.204, and 0.200 g CH_4-COD/g-VS/day, respectively) according to the authors' previous study (Li et al., 2017). These results indicated that a high methanogenic activity in the mesophilic CSTR co-digestion with WAS and FW can be realized even under high loading and low SRT conditions through the low-magnitude loading increasing regimes. The excellent performance of co-digestion with WAS and FW in mesophilic CSTRs under the high loading conditions in this study was closely related to the high hydrogenase and methanogenic enzyme activity of microbes, as shown in Fig. 4c. Protease, with an activity of 3645 U/L, was the dominant hydrolysis enzyme, the protease activity was approximately 99 times higher than the activity of α-glucosidase (α-glu, 36.8 U/L) in the mesophilic CSTR at an OLR of 53.5 g-COD/L/day. High protease activity enhanced the protein degradation efficiency and further improved the hydrolysis and methane yield. Furthermore, the high activity of coenzyme F_{420} (853 U/L) indicated an enriched methanogen biomass with a large VFA convention capacity even under a short SRT of approximately 2.5 days, which was maintained as the OLR increased from 24.1 to 52.1 g-COD/L/day (Fig. S2). Furthermore, the contents of acidogenic enzymes, including phosphotransacetylase (PTA), acetate kinase (AK), phosphotransbutyrylase (PTB), butyrate kinase (BK), [FeFe] hydrogenase (Hase), carbon monoxide dehydrogenase (CODH), and coA-transferase (CoA), determined the final VFAs, which have a close relationship with the metabolic pathways in mesophilic co-digestion with WAS and FW, as discussed in Section 3.4. In addition, to analyze the relationship between AD enzymes and co-digestion performance, the changes in AD enzymes during the whole experimental period need further investigation in the near future.

3.3. Microbial community characteristics

3.3.1. Bacterial community

The species and abundance of bacteria have a close relationship with the hydrolytic and acidogenic capacity to degrade complex organic matter during the AD process (Venkiteshwaran et al., 2017). The high activity and rapid proliferation of the bacterial community was the basis to realize a high methane yield and high-rate mesophilic CSTR digestion under low SRT conditions. As shown in Table 2, a similar Shannon index can be continuously maintained in the mesophilic CSTR even as the OLR increased to 52.1 g-COD/L/day and the SRT decreased to 2.56 days, indicating that a robust bacterial community was formed with constant bacterial diversity after long-term operation through the low-magnitude loading increasing regime. However, the ACE/Chao1 estimator gradually decreased as the SRT decreased to 2.56 days, indicating that the bacterial richness decreased because a large amount of microbial biomass was washed out under low SRT conditions. Meanwhile, a high concentration of TVFA of approximately 12.2 g-COD/L was discharged (Fig. 1c), and a high hydrolysis conversion ratio of approximately 33.8% was continuously maintained, as shown in Fig. 4a. These results suggested that the degraded function of the bacterial community in the present study was robust for the subsequent methanogenesis process under high load and low SRT conditions.

To adapt to the new conditions, large shifts in the bacterial community occurred after long-term operation compared to seed sludge, and similar phyla of the bacterial community were maintained as the OLR increased from 24.1 to 52.1 g-COD/L/day (Fig. 5a). During the high-rate period from Day 411 to Day 594, *Bacteroidetes* (9.14–40.0%) and *Firmicutes* (42.8–85.3%) were identified as the dominant bacterial phyla, which was in accordance with a previous report with the same feedstock and reactor structure under a lower OLR of less than 25.1 ± 1.8 g-COD/L/day (Li et al., 2018). As shown in Fig. 5a, the phylum *Bacteroidetes*, the dominant bacteria in the hydrolysis and acidogenesis process of the mesophilic CSTR co-digestion with WAS and FW, increased from an initial relative abundance (RA) of 0.09% to 9.14% and then evidently increased to 40.1% as the OLR increased, and the RA of the phylum *Bacteroidetes* reported in the present study was similar to that reported by Li et al. (2018), with a value of approximately 44.5%. On the other hand, the phylum *Firmicutes* (13.4–85.3%) was the common major phylum and appeared in the whole process for the mesophilic CSTR, and the RA of *Firmicutes* reported in this study was significantly higher than that reported by Li et al. (2018), with a value of approximately 7.48%. *Clostridia* (13.0–85.0%) was the predominant bacterial class affiliated with the phylum *Firmicutes*. Peng et al. (2018) demonstrated that *Clostridia* can produce cellulases, proteases, and other extracellular enzymes. In addition, the hydrolytic (α-glu and protease) and acidogenic (PTA, AK, PTB, BK, Hase, CODH, and CoA) enzymes in the mesophilic CSTR were all improved when the initial and end periods were compared (data not shown). These results indicated that a high RA of the phylum *Firmicutes* was beneficial for secreting a large amount of hydrolytic and acidogenic enzymes, realizing a high hydrolysis efficiency and achieving a high methane yield in the high-rate mesophilic CSTR co-digestion with WAS and FW. Additionally, the microbial community in the digestate under an OLR in the range of 30.7–38.8 g-COD/L/day from Day 505 to Day 545 was analyzed through metagenomic sequencing technology, as shown in Fig. 6. The taxonomic tree results were consistent with the high-throughput sequencing results and further defined the microorganisms to the species level in the high-rate mesophilic CSTR co-digestion with WAS and FW.

3.3.2. Archaeal community

To achieve the stable operation of high-rate AD processes, a robust archaeal community structure and methanogenesis species can result in a high VFA conversion rate, which is a crucial factor to avoid acid accumulation and methanogenesis inhibition and to maintain the balance among the hydrolysis, acidogenesis, acetogenesis, and methanogenesis steps. The OTUs and Shannon index both decreased during the whole experimental process (Table 2), indicating that the archaeal community diversity gradually decreased under high-rate mesophilic CSTR co-digestion with WAS and FW. Moreover, the ACE and Chao1 estimators both decreased and then increased, suggesting that the methanogenesis richness in the high-rate mesophilic CSTR under low SRT can still be achieved. As shown in Fig. 5b, the archaeal community structure was

Table 2
Biodiversity estimation of bacterial and archaeal communities in inoculum and digestate sludge from the mesophilic CSTR.

	Parameters	Sequence number	OTUs	Shannon	Simpson	ACE	Chao1	Coverage
Bacteria	Inoculum	71,964	1722	2.29	0.23	67,268	23,475	0.979
	Day 411 (OLR = 24.1 g-COD/L/d; SRT = 5.56 d)	83,672	1997	2.19	0.32	30,978	13,134	0.981
	Day 594 (OLR = 52.1 g-COD/L/d; SRT = 2.56 d)	80,505	1981	2.52	0.24	18,283	9227	0.982
Archaea	Inoculum	152,373	2323	0.61	0.79	413,516	87,261	0.985
	Day 411 (OLR = 24.1 g-COD/L/d; SRT = 5.56 d)	50,348	435	0.45	0.89	20,675	7085	0.992
	Day 594 (OLR = 52.1 g-COD/L/d; SRT = 2.56 d)	44,481	425	0.28	0.94	36,846	11,598	0.991

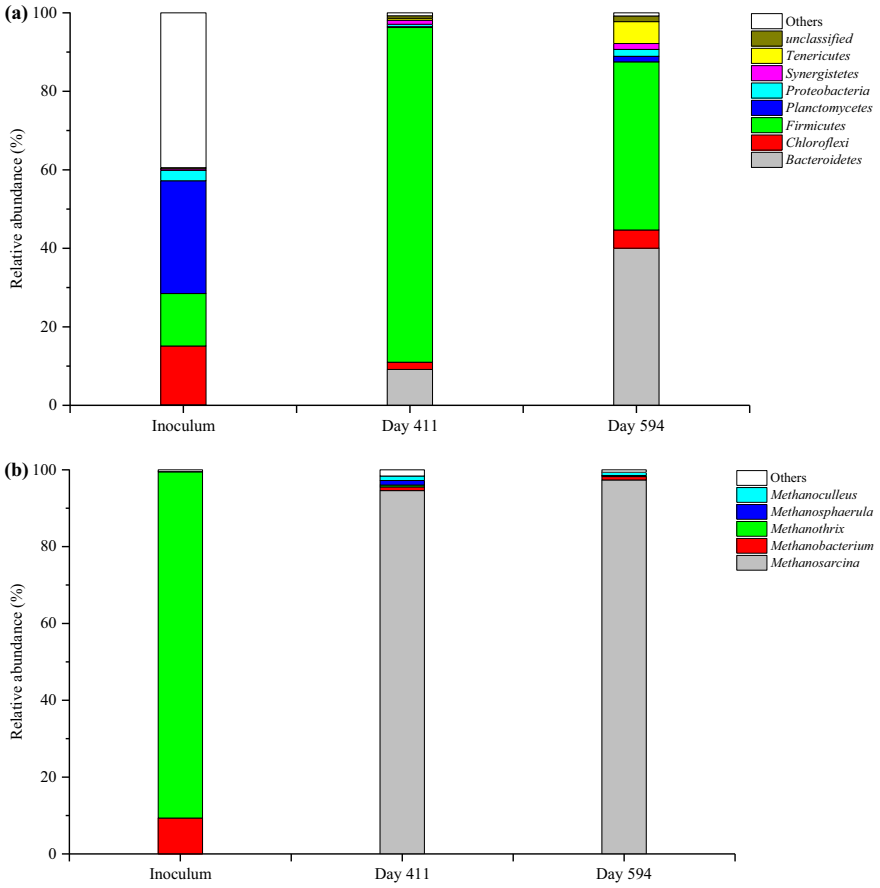

Fig. 5. Similarity and RA variation of bacteria (a) and archaea (b) in inoculum and digestate sludge on Day 411 and Day 594.

shifted, changing from acetotrophic methanogens (*Methanothrix*, 90.1%) and hydrogenotrophic methanogens (*Methanobacterium*, 9.32%) towards a *Methanosarcina* (94.6%) dominated community with an elevated OLR. Obviously, *Methanosarcina* was one of the most dominant methanogenic communities in the mesophilic CSTR co-digestion system. Then, the RA of *Methanosarcina* was further improved from 94.6% to 97.3%, and the SRT decreased from 5.56 to 2.56 days. *Methanosarcina* has been reported to produce methane via all pathways utilizing acetate and H_2 combined with CO_2 (Chen et al., 2018). Compared to other methanogens, *Methanosarcina* has high growth rates and is quite robust towards different impairments (De Vrieze et al., 2012). As shown in Fig. 6, *Methanosarcina barkeri* and *Methanosarcina flavescens* were the two predominant species belonging to the *Methanosarcina* genus; both species have acetoclastic and hydrogenotrophic methane-producing pathways (De Vrieze et al., 2012; Shin et al., 2019). In addition, *Methanosarcina* species have also been demonstrated to participate in direct interspecies electron transfer (Li et al., 2020c; Lovley, 2017). Therefore, the high RA of *Methanosarcina* is crucial for the stable operation of high-rate mesophilic CSTR co-digestion at low SRTs. In addition, the discharge from the high-rate mesophilic CSTR can be used as inoculation to rapidly start-up high-rate digestion and even used as bioaugmentation to recover the inhibited digesters or to improve the stable operation of the AD system, which will be further investigated in the near future.

3.4. Metabolic pathway of high-rate mesophilic CSTR co-digestion

Explicitly identifying the species of the microbial community using metagenomic approaches was beneficial to further explain the metabolic pathways in the high-rate mesophilic CSTR co-digestion using low-magnitude loading regimes. Based on the metagenomics analysis, AD enzymes in combination with the main metabolic pathways of the AD processes (Bi et al., 2020; Chen et al., 2020; Li et al., 2019), the microbial metabolic pathway in the high-rate mesophilic CSTR co-digestion with WAS and FW was proposed and constructed as shown in Fig. 7. Hydrolytic bacteria are phylogenetically diverse phyla belonging to *Firmicutes*, *Bacteroidetes*, and *Chloroflexi*, which can decompose substrates into SCODs, including proteins and polysaccharides, and then degrade them into monosaccharides with microbial proteases and α-glu. The acidogenic bacteria are involved in the second step of co-digestion, which converts monosaccharides into VFAs, CO_2, and H_2 with PTB, BK, and Hase. The acidogenic bacteria include *Actinomyces* sp. *S4-C9*, *Actinomyces europaeus*, and *Candidatus Cloacimonas acidaminovorans*. Acetogens play a key role in the third stage in establishing the syntrophic association between acetogens and methanogens (Mehariya et al., 2018). The acetogenic bacteria include *Proteiniphilum acetatigenes* and *Faecalibacterium* sp. *CAG:74* and convert n-butyrate and H_2/CO_2 to acetic acid with PTA, AK, CoA, and CODH. The genus *Methanosarcina* provides metabolic capability in both acetoclastic and hydrogenotrophic methanogenesis and has also been reported to be more favorable in elevating FA and VFAs (De Vrieze et al., 2012; Lins et al., 2014). In the final step, *Methanosarcina barkeri* and *Methanosarcina flavescens* were established as the main methane formation pathways with high coenzyme F_{420} activity in the high-rate mesophilic CSTR co-digestion with WAS and FW.

In general, the volumes of conventional digesters are large due to a long-term retention time required, making the footprints are substantial (Cheng et al., 2020). The high-rate and stable mesophilic CSTR co-digestion with WAS and FW at low SRT of 2.56–2.63 days was realized through a low-magnitude loading regime in the present study. Low-magnitude loading regimes give a low loading shock and adequate time for AD microbial shifts for adaptation to high-rate operation

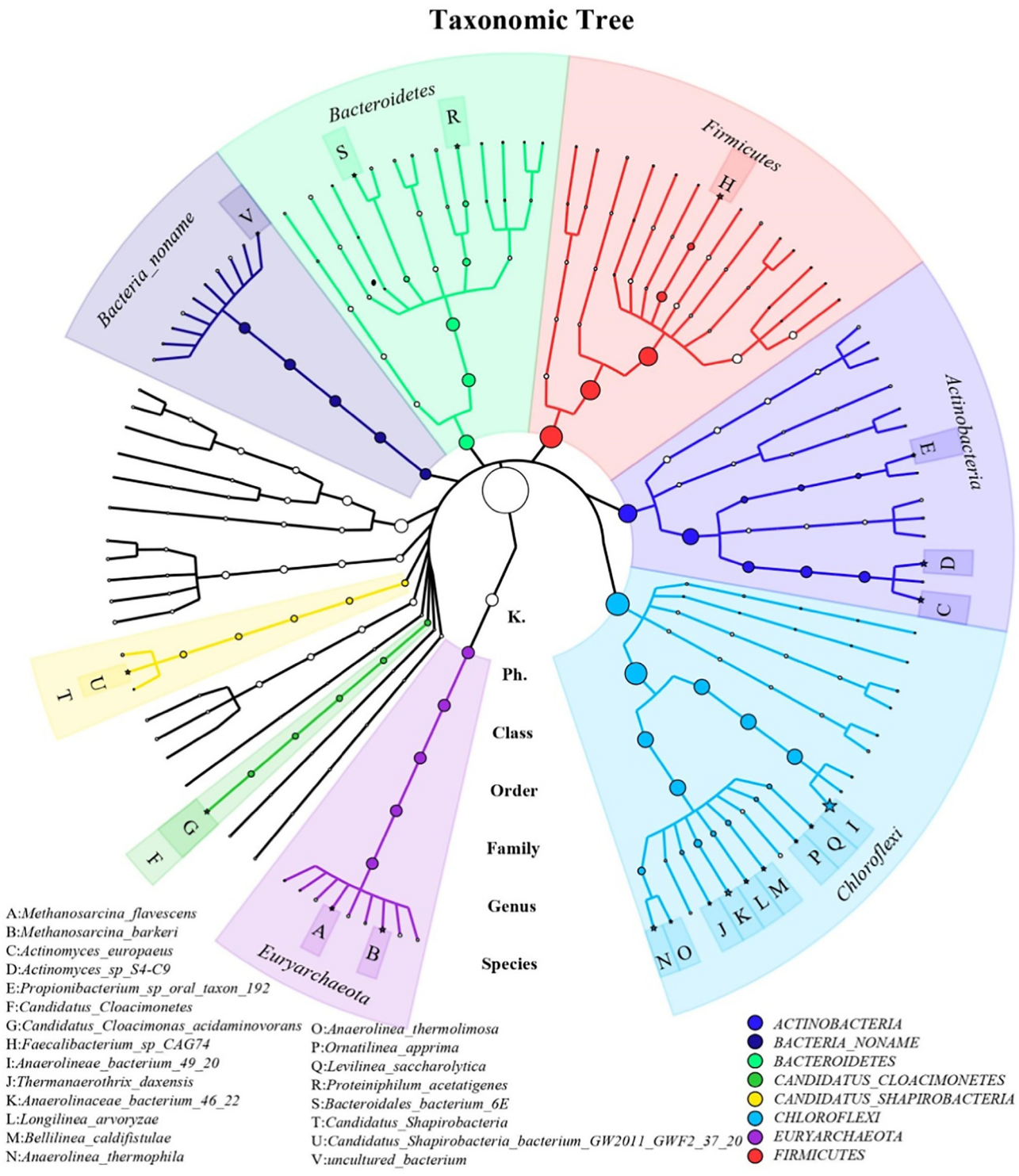

Fig. 6. Taxonomic tree in the digestate sludge under OLRs in the range of 30.7 to 38.8 g-COD/L/d from Day 505 to Day 545 through metagenomics analysis.

conditions. The genus *Methanosarcina* was enriched in the mesophilic CSTR with a high activity of AD enzymes and SMA; this avoids VFA accumulation and further realizes a high methane yield and methane production, which was the main reason for the stable operation of the high-efficiency mesophilic CSTR co-digestion with WAS and FW. These results suggested that the conventional CSTR co-digestion efficiency can be further improved without additional changes. Furthermore, in a real AD operating at 2.56–2.63 days instead of 20 days, this could suppose a reduction in costs for the AD of biowaste. In addition, based on the high-rate mesophilic CSTR co-digestion through the low-magnitude loading regime, changing the reactor structure from a CSTR to an anaerobic membrane bioreactor can achieve a higher loading mesophilic co-digestion by an extent SRT larger than 2.5 days and reduce the HRT to less than 2.5 days (data not shown). Thus, realizing *Methanosarcina* enrichment through a low-magnitude loading regime is a promising method to realize the stable operation of high-efficiency co-digestion for biogas production in practical engineering applications.

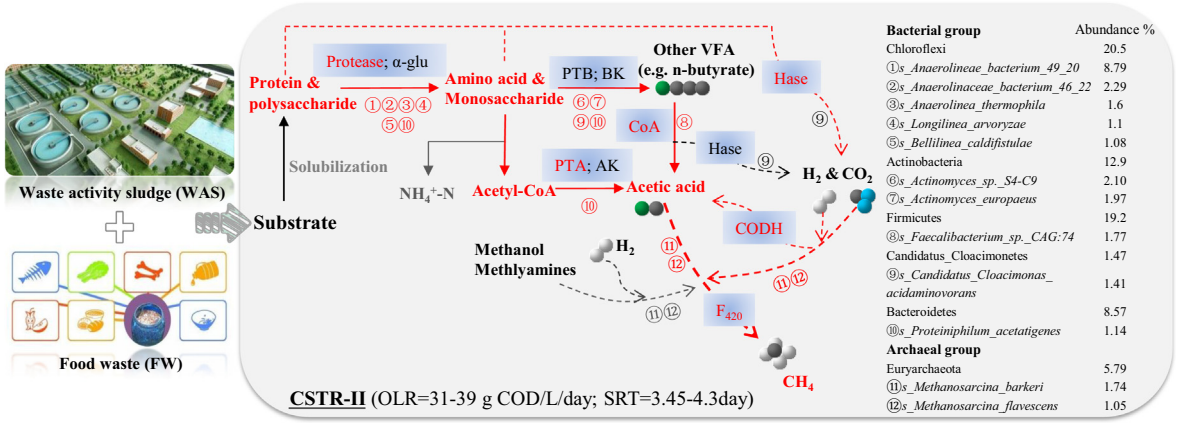

Fig. 7. A schematic diagram of metabolic pathways of the mesophilic co-digestion of WAS and FW in a high loading rate CSTR.

4. Conclusions

High-rate and stable operation of mesophilic CSTR co-digestion with WAS and FW can be realized after long-term acclimatization through the low-magnitude loading increase regimes in this study. The maximum methane production of 12.9 L/L/day was realized with an OLR of 48.1 g-COD/L/day and an SRT of 2.78 days. Furthermore, the mesophilic CSTR co-digestion was still stable at SRT of 2.56–2.63 days without a large amount of VFA accumulation, and a high methane yield, hydrolysis conversion ratio, methanogenic activity, and AD enzymes were all maintained during the high-rate operation period with OLR of 50.8–52.1 g-COD/L/day. The main advantages of the low-magnitude loading regime were the low loading shock and long-term acclimatization for AD microorganisms to adapt to the high-rate operation conditions. The high abundance of the class *Clostridia* (13.0–85.0%) ensured a high hydrolysis rate, which increased the co-digestion degradation degree of the substrate mixture. The high abundance of the genus *Methanosarcina* (94.3–97.3%) secreted a large amount of coenzyme F_{420} content and presented high methanogenic activity with individual VFAs and H_2/CO_2. The low-magnitude loading regime is a promising method for achieving the enrichment of *Methanosarcina*, which is crucial for the stable operation of high-efficiency co-digestion for biogas production at low retention times.

CRediT authorship contribution statement

Bao-Shan Xing: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **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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Appendix A. Supplementary data

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