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Cow manure as additive to a DMBR for stable and high-rate digestion of food waste: Performance and microbial community

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ABSTRACT

Cow manure (CM) was added to a dynamic membrane bioreactor (DMBR) operated under anaerobic condition for enhancing food waste (FW) digestion for over 300 days with stepwise increase of organic loading rates (OLRs) from 1.07 to 11.9 g COD/L/day. At a FW/CM ratio of 3.5:1 (based on volatile solids), the mixed liquor pH was always above 8.0 and no apparent volatile fatty acids (VFAs) accumulation occurred even at the highest OLR of 11.9 g COD/L/day (hydraulic retention time as 10 days and solid retention time as 15.5 days, correspondingly), indicating a very stable operation condition which resulted in an average CH_4 yield as high as 250 mL/g COD and CH_4 production as high as 2.71 L CH_4/L/day. The hardly biodegradable organic components, such as cellulose, hemicellulose, and lignin, were effectively degraded by 78.3%, 58.8%, and 47.5%, respectively. Significantly high anaerobic digestion reaction ratios, especially the hydrolysis ratio which is usually the limiting factor, were calculated based on experimental results. Furthermore, the high lignocellulase contents and coenzyme F_{420} levels, along with the decrease of cellulose crystallinity from 72.6% to 16.4% in the feedstock, provided strong evidence of an enhanced biological activity by CM addition. By high-throughput sequencing analysis, more abundant and diverse bacterial, archaeal, and fungal genera were identified from the DMBR sludge. With CM addition, the biodegradation of lignocellulose might have produced sufficient H_2 and CO_2 for the hydrogenotrophic methanogens such as *Methanoculleus*, *Methanomassiliicoccus*, and *Methanobacterium*, which were highly tolerant to ammonium inhibition, and then the elevated ammonium level would have provided high buffering capacity in the DMBR thus ensuring a stable condition for high rate FW digestion and CH_4 production.

1. Introduction

With the rapid development of the modern catering industry, food waste (FW) production is increasing rapidly. The FW production of urban areas has been predicted to increase by 44% from 2005 to 2025 due to economic and population growth, particularly in developing countries (Melikoglu et al., 2013). FW is mainly composed of carbohydrate polymers (starch, cellulose, hemicellulose), lignin, proteins, lipids, organic acids, and a smaller, inorganic part (Xiao et al., 2018; Zhang et al., 2014) and is an easily biodegradable organic substrate for anaerobic digestion (AD) due to its high potential for bio-methane production (Dahiya et al., 2018; Neves et al., 2009). However, inhibition has always occurred when FW was digested alone at high organic loading rates (OLRs) (Zhang et al., 2014). To counteract the inhibition and to improve the performance of digesting FW alone, wastewater (Brown and Li, 2013; Rajagopal et al., 2013), waste activated sludge (Li et al., 2017a, 2018a), and paper waste (Qin et al., 2018) have been used as a co-substrate in batch and semi-continuous tests. The large amount of cow manure (CM) that exists has been continuously increasing as a result of livestock industry growth (Tišma et al., 2018). Due to the high buffering capacity and nutrient balance of FW digestion along with the addition of CM, the methane yield and system stability of FW digesters were improved (El-Mashad and Zhang, 2010; Li et al., 2009; Marañón et al., 2012; Zhang et al., 2013). Zhang et al. (2013) found that the digestion of FW with CM not only improved the

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maximum acceptable organic loading rates but also promoted the methane yield in semi-continuous digestion. Li et al. (2009) obtained a 44% improvement in the methane yield by adding CM to digestion of FW. Digesting most organic wastes with FW improves the efficiency of methane production and the stability of the system. Adding CM to AD of FW may enhance the stability of the anaerobic process mainly due to an increased carbon to nitrogen (C/N) balance and an increased buffer capacity, which has been reported by previous studies (El-Mashad and Zhang, 2010). As we know, the balance of the microbial community can also result in process stability, and the microbial community structure is also shaped by substrate and operating parameters. To date, no study has been carried out to address the microbial community shift in FW digestion when CM is added.

The bioenergy conversion efficiency of digesting FW alone is not ideal because FW has a long hydraulic retention time (HRT) of more than 20 days, a low OLR of 1–6 g VS/L/day, and a low bioenergy conversion rate of 40–70% (Ma et al., 2018). A stable and high-rate digestion of FW for efficient bioenergy conversion has significantly correlation with the applied mode (batch or continuous), reactor type, whether digestion is performed in one or two stages, and operating parameters (temperature, OLR, HRT and solid retention time (SRT)). Compared with two-phase AD, single-stage digestion requires a lower degree of control and less space, which may be the reason why there are few examples of two-stage processes run on a commercial scale (Schnürer, 2016). For a single-phase system, stable operation and a high substrate load can further reduce the occupied space. A higher load requires a high biomass activity and a large biomass amount. Use of an anaerobic membrane reactor (AnMBR) is considered an efficient method for methane generation from solid waste and wastewater (Li et al., 2015). HRT and SRT in AnMBR can be independently controlled to extend process applicability for treating wastewaters and solid wastes. However, the intensive use of AnMBR is limited by several critical obstacles, such as low flux, membrane fouling, and high capital and operation costs. Dynamic membrane (DM) technology offers an innovative way to address these issues associated with conventional membrane technology (Tang et al., 2017). Cayetano et al. (2019) investigated the applicability of external DM technology in the anaerobic treatment of FW for biomethane recovery. Their relatively high biomass retention enabled the digestion of FW with CH_4 production rates of up to 1.2 L/L/day under an OLR of 5.0 g COD/L/day. Therefore, it is believed that by combining the positive effects of CM and dynamic membrane bioreactor (DMBR) on FW degradation and slow-growing bacteria, stable methane fermentation may be achieved under higher OLRs.

Although CM addition has been found effective to improve FW digestion efficiency in the aforementioned studies, to our knowledge, the underlying mechanisms are still unclear, especially the system characteristics in long-term operation and microbial community features. Therefore, the main objective of this study was to reveal the characteristics of biogas production using a DMBR with FW as main substrate and CM as additive. Attentions were paid to the system stability under varied OLRs in long-term operation, and the associated microbial community covering bacteria, archaea and fungi.

2. Materials and methods

2.1. Feedstock and inoculum

The FW was manually prepared based on the characteristics of FW in China. Details about the processing procedures of FW are given in the authors' previous study (Li et al., 2017a). The FW consisted of cabbage (20%), pork meat (10%), chicken meat (5%), egg (5%), cooking oil (1%), potato (20%), carrot (13.8%), rice (15%), noodles (10%), and table salt (0.2%), all based on wet weights. CM was obtained from a rural area near Xi'an, China. To obtain higher methane yields, the FW and CM were mixed at the optimum FW/CM ratio of 3.5:1, which was based on volatile solids (VS) content and determined from preliminary experimental results (data not shown). The mixture was then crushed for 10 min using a blender and diluted with tap water to obtain feedstock with a TS content of approximately 7.0%. The inoculum sludge was taken from a full-scale mesophilic anaerobic reactor of a brewery plant in Xi'an, China. The physicochemical characteristics of the FW, CM, and inoculum used in this study were determined, and they are presented in Table 1.

2.2. Reactor configuration, operation, and experimental procedure

A long-term experiment was conducted via a DMBR with a working volume of 0.7 L, as shown in Fig. 1. A submerged filtration module made of a nylon mesh with an equivalent aperture of 50 μm was used to support the growth of the DM (cake layer). The filtration module had a surface area of 14.4 cm^2 (two parallel filtering surfaces of 3.8 × 3.8 cm). A water jacket and thermostatically controlled water baths were used to control the temperature of the reactor under mesophilic conditions (39 °C). Feedstock was pumped from a substrate tank to the DMBR by a peristaltic pump that was maintained at 4 °C. A digital pressure meter (SIN–P300, Sinomeasure, China) was installed between the membrane module and the effluent extraction pump to record the trans-membrane pressure (TMP). The final pressure increased gradually with time in constant flux operation mode. When the final pressure increased to 16 kPa, a physical cleaning method (biogas backwashing with a flow rate of 10 L/min for 2 min) was applied for permeability recovery. The experiment included ten stages, which divided by different HRTs from 100 to 10 days as showed in Table 2. For a stable start-up, the reactor was initially seeded with 0.7 L of seed sludge and fed at a low OLR of 1.07 g COD/L/day (Stage 1). After the initial stage which lasted for 47 days, HRT was shortened gradually in the manner indicated in Table 2. For accurate HRT control, a suction pump was operated under prescribed on/off frequency for

Table 1
Physicochemical characteristics of food waste (FW), cow manure (CM), and inoculum.

Parameter	FW	CM	Inoculum
TS (g/L)	136.9 ± 16.1	120.8 ± 9.1	23.9
VS (g/L)	130.8 ± 15.2	104.5 ± 3.9	12.1
TCOD (g/L)	223.9 ± 8.6	100.9 ± 15.5	18.4 ± 0.5
SCOD (g/L)	122.8 ± 5.5	20.0 ± 2.1	0.16 ± 0.01
pH	4.34	7.08	7.33
Protein (g/L)	3.07 ± 0.03	3.80 ± 0.12	0.034 ± 0.002
Carbohydrate (g/L)	82.1 ± 1.5	1.36 ± 0.02	0.028 ± 0.001
NH_4^+-N (mg/L)	312.2	376.5	44.3 ± 1.2
Alkalinity (g $CaCO_3$/L)	ND	13.5	12.5
Acetic acid (mg/L)	1850.9	69.8	13.4
Propionic acid (mg/L)	ND	14.7	1.4
Butyric acid (mg/L)	ND	3.8	ND
C (%)	47.8 ± 0.53	40.1 ± 0.54	/
H (%)	4.72 ± 0.13	5.66 ± 0.19	/
O (%)	27.3 ± 1.18	35.2 ± 0.47	/
N (%)	5.71 ± 0.05	2.01 ± 0.01	/
S (%)	0.87 ± 0.04	0.67 ± 0.02	/
Cellulose (%TS)	5.7	16.9	/
Hemicellulose (%TS)	2.4	15.6	/
Lignin (%TS)	1.8	25.1	/

Notes: ND means not detected; "/" means not applicable.

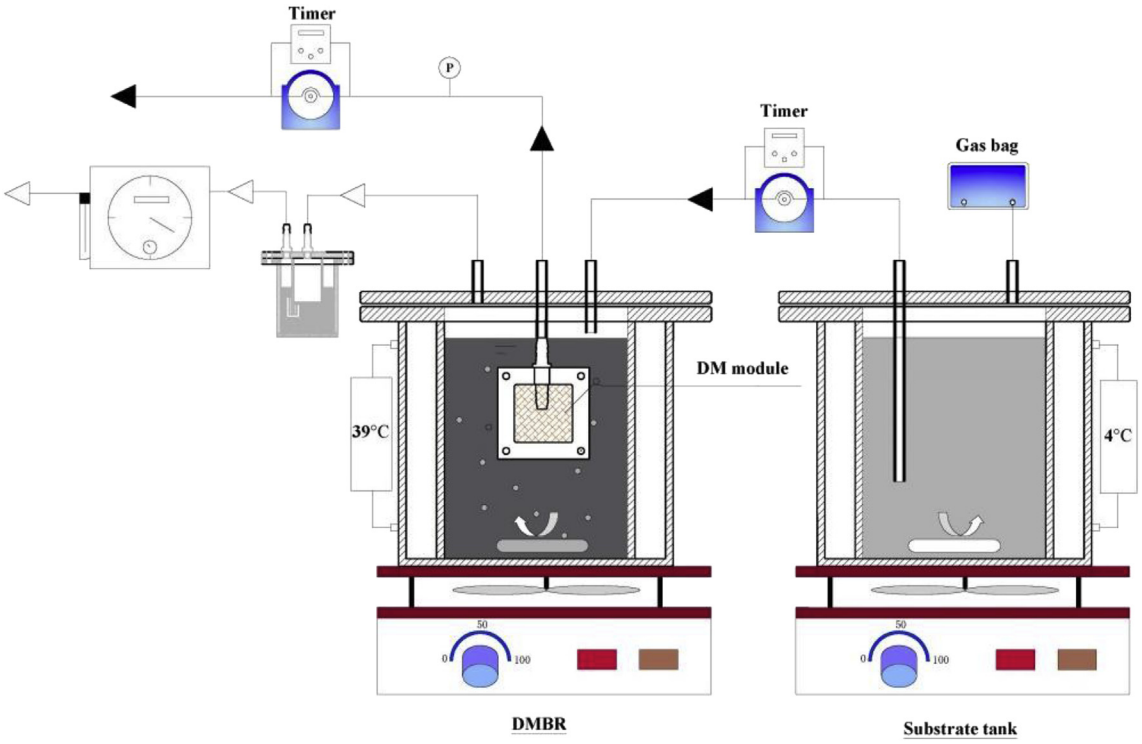

Fig. 1. Schematic diagram of the submerged dynamic membrane bioreactor (DMBR) setup and operation.

Table 2
Performance of anaerobic mesophilic digestion of FW with CM as additive in a semi-continuous DMBR.

Parameter		Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	Stage 7	Stage 8	Stage 9	Stage 10
HRT	day	100	50	33.3	25	20	16.7	14.3	12.5	11.1	10
SRT	day	159	80	53	40	32	26.6	22.7	19.9	18.1	15.5
Cycle numbers	/	1	2	3	4	5	6	7	8	9	10
OLR	g TS/L/day	0.7 ± 0.1	1.4 ± 0.1	2.1 ± 0.1	2.8 ± 0.1	3.6 ± 0.4	4.0 ± 0.1	5.2 ± 0.2	6.4 ± 0.1	6.6 ± 0.2	7.6 ± 0.6
	g VS/L/day	0.6 ± 0.1	1.3 ± 0.1	1.9 ± 0.1	2.6 ± 0.1	3.3 ± 0.4	3.7 ± 0.1	4.8 ± 0.2	5.9 ± 0.1	6.1 ± 0.2	7.1 ± 0.5
	g COD/L/day	1.07 ± 0.01	2.15 ± 0.01	3.22 ± 0.01	4.35 ± 0.18	5.57 ± 0.68	6.18 ± 0.19	8.04 ± 0.37	9.98 ± 0.18	10.3 ± 0.28	11.9 ± 0.87
Duration	days	1–47	48–74	75–87	88–116	117–130	131–150	151–205	206–244	245–271	272–312
CH_4 production	mL/L/day	110 ± 39	568 ± 131	899 ± 131	1088 ± 205	1517 ± 189	1860 ± 222	1982 ± 255	2129 ± 283	2751 ± 222	2708 ± 317
COD removal rate	g COD/L/day	0.31 ± 0.11	1.62 ± 0.37	2.57 ± 0.37	2.68 ± 0.61	3.93 ± 0.57	4.55 ± 1.09	5.14 ± 0.74	5.50 ± 0.82	7.21 ± 0.92	7.02 ± 1.02
CH_4 yield	mL CH_4/g COD	103 ± 36	270 ± 60	280 ± 41	253 ± 57	272 ± 52	287 ± 42	245 ± 32	209 ± 26	264 ± 36	250 ± 31
pH_{output}	/	8.2 ± 0.2	7.7 ± 0.2	7.9 ± 0.2	8.1 ± 0.2	8.2 ± 0.1	8.1 ± 0.1	7.9 ± 0.3	8.1 ± 0.2	8.4 ± 0.2	8.4 ± 0.1
NH_4^+-N	g/L	0.18 ± 0.03	0.51 ± 0.17	0.77 ± 0.27	0.80 ± 0.28	1.14 ± 0.41	1.30 ± 0.47	1.45 ± 0.52	1.88 ± 0.55	1.97 ± 0.72	1.38 ± 0.69
Acetic acid	mg COD/L	8.3 ± 1.4	9.1 ± 5.8	10.9 ± 3.6	18.2 ± 16.3	10.5 ± 3.0	8.5 ± 2.6	6.7 ± 2.2	7.0 ± 3.7	8.5 ± 11.5	4.1 ± 3.1
Propionic acid	mg COD/L	2.5 ± 2.5	1.5 ± 2.2	2.1 ± 2.3	6.6 ± 10.0	0.9 ± 1.8	1.4 ± 1.7	0.2 ± 0.6	0.3 ± 1.0	0.7 ± 2.6	0.0 ± 0.0
Butyric acid	mg COD/L	0.8 ± 1.3	1.2 ± 2.0	0.3 ± 0.5	5.6 ± 12.2	0.9 ± 1.8	0.7 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.7	0.0 ± 0.0
Valeric acid	mg COD/L	1.9 ± 2.9	0.4 ± 1.2	1.9 ± 1.8	6.5 ± 10.7	1.7 ± 2.4	1.5 ± 1.8	0.1 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
TVFA	mg COD/L	12.7 ± 6.4	11.1 ± 7.6	14.9 ± 7.3	31.3 ± 32.7	13.1 ± 5.7	11.3 ± 5.1	7.0 ± 2.4	7.3 ± 4.2	9.2 ± 14.1	4.1 ± 3.1

Note: Cycle number, the frequency of effluent/influent every day; CH_4 yield, calculated according to CH_4 production and OLR applied for operation condition.

extracting effluent from the reactor. In each operation cycle, 7 mL effluent was extracted and the same volume of feedstock was added to the reactor. With increasing frequency of pump operation, both OLR and HRT were controlled and maintained in each stage as indicated in Table 2, until the final stage (Stage 10) where OLR was increased to 11.9 g COD/L/day and HRT was decreased to 10 days. The filtration performance metrics of the DM membrane, such as solid interception levels, fouling rate and associated cleaning frequency, were then determined accordingly. Meanwhile, the reaction ratios and enzyme contents of DMBR sludge and cellulose, hemicellulose, lignin, and the microbial community of feedstock and DMBR sludge were sampled and investigated, and X-ray diffraction (XRD) and Fourier transform-infrared (FTIR) spectroscopy were applied.

2.3. Lignocellulose-degrading enzyme and coenzyme F_{420} content

Samples for lignocellulose-degrading enzyme were centrifuged at 12000 g for 15 min, and then the supernatant was filtered using a syringe membrane with a 0.45 μm pore size. The sample was diluted with deionized water to obtain absorbance readings in a linear measurement range, and these readings were converted to activity in U/mL. The lignocellulolytic enzymes that were analysed were lignin peroxidase (LiP), manganese peroxidase (MnP), laccase (Lac), xylanase, carboxymethyl cellulase (CMCase), xylitolase, xylan esterase, β-glucosidase (BG), endoglucanase (EG), and cellobiose hydrolase (CBH). The contents of lignocellulose-degrading enzymes were measured by enzyme-linked immunosorbent assay (ELISA) kits (MSKBIO, Wuhan, China). Absorbance was measured by a

multiscan spectrophotometer (Varioskan™ LUX, Thermo Fisher, Finland) at 450 nm. The concentration of conenzyme F_{420} in DMBR sludge was determined according to the methods described by Reynolds and Colleran (1987).

2.4. Microbial community analysis

Samples were collected from seed sludge, feedstock, and the DMBR on day 262 to characterize the diversity of microbial communities via high throughput sequencing technology. DNA was extracted with 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 the forward primer 341F (5′-CCTACGGGNGGCWGCAG-3′) and the reverse primer 805R (5′-GACTACHVGGGTATCTAATCC-3′) for bacteria and primers 349F (5′-GYGCASCAGKCGMGAAW-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) for archaea. PCR targeting the 18S rRNA gene was performed using the primers Fung (5′-ATTCCCCGTTACCCGTTG-3′) and NS1 (5′-GTAGTCATATGCTTGTCTC-3′) for eukaryota. After being purified and quantified, the PCR products of the V3—V4 region of the 16S rRNA gene and the NS1-fung region of the 18S rRNA gene were sequenced using the Illumina HiSeq 2000 sequencer (Sangon Biotech Shanghai Co., Ltd., China). The obtained sequence fragments were assembled using Flash software. Rarefaction curves and the Shannon diversity index, Chao1 species richness estimator and coverage index were calculated by MOTHUR to identify the species diversity for each sample (Zhang et al., 2017). UCHIME was then used to remove chimaeric sequences, and sequences with more than 97% similarity were clustered to form operation taxonomic units (OTUs). The ribosomal database project was used for alignment at a confidence threshold of 80% (Wang et al., 2007).

2.5. Analytical methods

The content of soluble chemical oxygen demand (SCOD), total chemical oxygen demand (TCOD), total solids (TS), VS, alkalinity, protein, carbohydrate, and NH_4^+-N were analysed based on standard methods (APHA, 2005). Turbidity was measured by a portable turbidity meter (Turb® 355 IR, Xylem company, Germany), pH with a portable pH meter (Horiba, Kyoto, Japan), and the filtration flux of the DM with a volumetric method. The biogas production, composition of the various biogases (CH_4, CO_2, N_2 and H_2), and volatile fatty acid (VFA) levels were measured as described by Li et al. (2017a). The elemental composition of C, H, O, N and S were analysed using an elemental analyser (Vario PYRO cube; Elementar Company, Germany). Analysis of cellulose, hemicellulose, and lignin was conducted using an HPLC system (LC-20A; Shimadzu, Japan) with an Aminex HPX-87H column (300 mm × 7.8 mm) (Bio-Rad, USA) and a refractive index detector (RID-10A; Shimadzu, Japan) according to the method developed by Sluiter et al. (2008). The particle size distribution (PSDs) of the feedstock, cake sludge, and fermentation mixture were analysed using a laser granularity distribution analyser (LS 230/SVM+, Beckman Coulter Corporation, USA) with a detection range of 0.4—2000 μm. The morphological properties of the membrane and nylon mesh were observed by a scanning electron microscopy (SEM, MLA650 FEG, FEI, USA) analyser according to the methods described by Hu et al. (2016). The crystallinity of the raw materials and residues were studied using XRD. The crystallinity index is calculated from the ratio of the area of all crystalline peaks (101, 10$\bar{1}$, 021, 002 and 040) to the total area basis on the corresponding XRD patterns (Park et al., 2010). Chemical bond changes were studied by FTIR at 4000-400 cm^{-1}. The free ammonia concentration in the digestate was calculated based on equilibrium as proposed by Anthonisen et al. (1976).

3. Results and discussion

3.1. Long-term stable operation of FW digestion with CM as additive

3.1.1. Performance of the DMBR

AD was found to be unstable when FW or CM was used as mono-substrate (Zhang et al., 2013). Our preliminary experimental results (data not shown) indicated that CH_4 yield could be improved by 83% in comparison with the weighted average of mono-digestion of FW and CM, about 2 times of the CH_4 yield increase (44%) for the co-digestion of kitchen waste and CM in comparison with the mono-digestion of kitchen waste (Li et al., 2009). In order to achieve more stable operation and higher biogas production efficiency, CM was chosen as an additive for the FW digester in this study of long-term continuous operation with different OLRs in the DMBR (Table 2). Biogas production (GP), methane production (MP), methane content, VFAs and pH in the DMBR are shown in Fig. 2. According to the data shown in Table 2, the average CH_4 yield could be calculated as 408 mL CH_4/g VS, approximately equivalent to 92.5% of the theoretical CH_4 yield (441 mL CH_4/g VS) based on Buswell and Mueller (1952) and the elemental composition of FW and CM (Table 1). During Stage 1, the methane content increased with time, which possibly indicated that the microbial community was acclimated to gradually adapting the substrates and enrichment in the DMBR. Subsequently, the methane content of the biogas in the DMBR (Fig. 2(b)) approached the theoretical level (Buswell and Mueller, 1952), indicating that with CM as additive, the feed stock could be steadily converted to biogas. As shown in Fig. 2(c) and Table 2, the maximum total volatile fatty acid (TVFA) was only 170 mg COD/L, and the average output pH (pH_{output}) values changed in the range of 7.7—8.4 (Table 2) in the whole operation period. Furthermore, the high alkalinity concentrations were in the range of 2.1—2.9 g $CaCO_3$/L in the DMBR from day 274 to day 300. Therefore, the TVFA to alkalinity ratio was significantly lower than 0.4, which is considered the threshold for system stability; higher values indicate unstable operating conditions in the digester (Li et al., 2017a). It can be seen from Fig. 2(c), after day 150 only acetic acid was frequently measured at low level in the digester. These results suggest that the addition of CM to FW stabilizes the anaerobic system, in-line with previous findings (Zhang et al., 2013).

Generally, methanogenesis was severely inhibited after 250 mg NH_3/L was reached in reactors with unacclimated sludge (Yenigun and Demirel, 2013). However, stable reactor operation was achieved with 529 mg NH_3/L (1880 mg NH_4^+-N/L) and a pH value of 8.31 on day 244. At a pH of 8.14 and free ammonia concentration of 283 mg NH_3/L (1376 mg NH_4^+-N/L), the digester could also be operated satisfactorily on day 312. Therefore, it can be concluded that the FW digester can operate stably even with a high OLR (11.9 ± 0.87 g COD/L/day) and free ammonia along with long-term CM addition into the DMBR, which was consistent with Agyeman and Tao (2014). Typically, the nutrient balance and relatively high buffer capacity were considered the main reasons for the stability of FW and CM digestion. However, according to the report by El-Mashad and Zhang (2007), the FW digester showed unstable performance at an OLR of 4 g VS/L/day after 65 days of operation under an OLR of 2 g VS/L/day and a substrate mixture of 48% (based on VS) FW and 52% CM. The fluctuation may be due to the abundance and conversion ratio of the microbial community not being strong enough without long-term accumulation and/or very large increase in OLR. Meanwhile, only 71.3% methane production and less than 64.8% degradability were achieved after 10 days of using the same feedstock (FW/CM of 3.5:1, VS/VS) and inoculum (Table 1) at an organic loading of 7.1 ± 0.5 g VS/L in biochemical methane potential assays. However, stable digester performance and good biogas

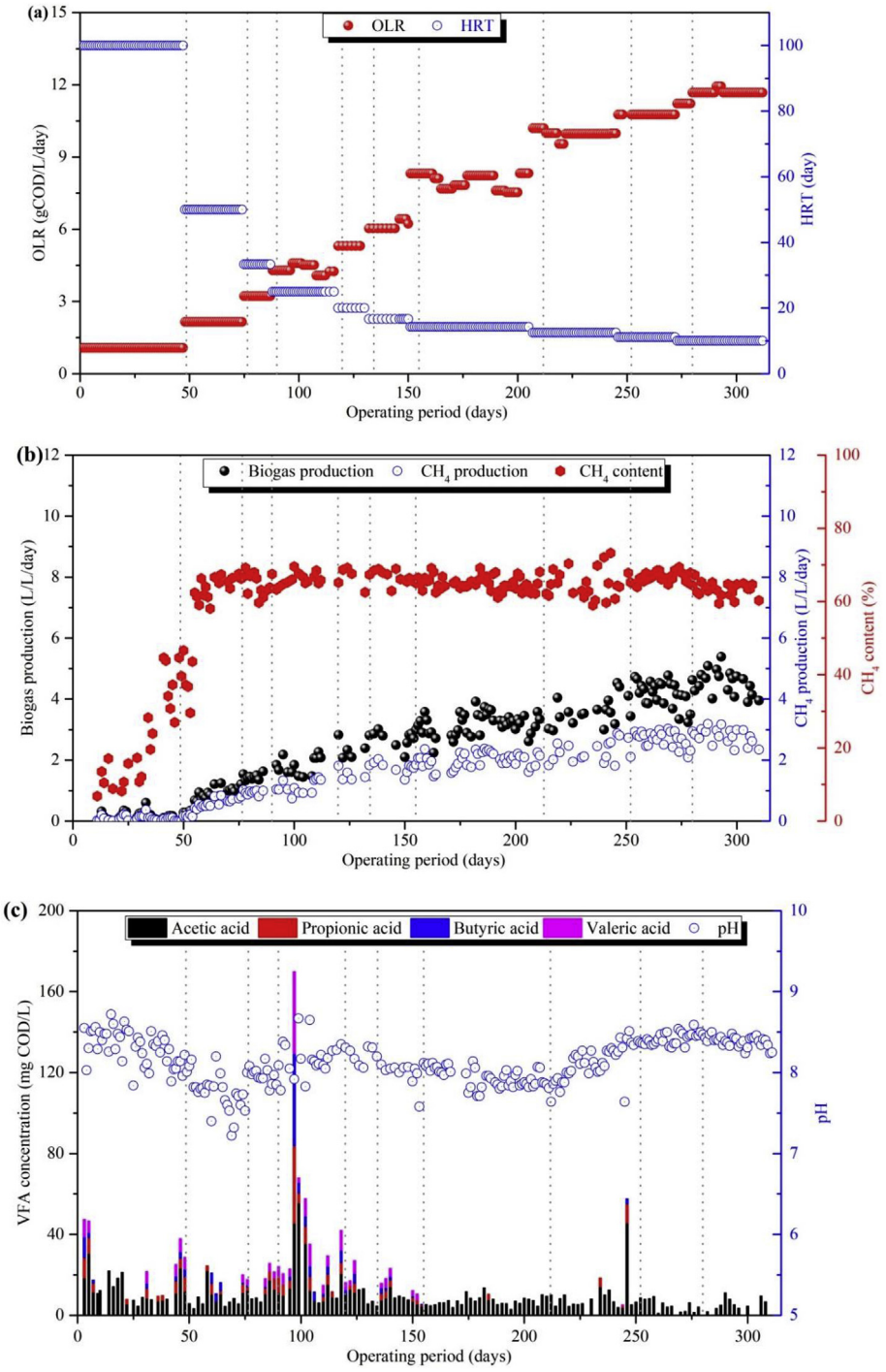

Fig. 2. Variation in (a) organic loading rate (OLR) and hydraulic retention time (HRT); (b) biogas production (GP), methane production (MP) and methane content; (c) VFAs and pH in the DMBR throughout the process.

production rate and yield were obtained at an OLR of 7.1 ± 0.5 g VS/L/day and an HRT of 10 days. Those results indicated that the long-term microbial community accumulation and bioaugmentation with sequential biocatalyst addition may be considered the main reason for the DMBR performance stability.

3.1.2. *Performance of DM module*

The stability in the DM performance is an important prerequisite for the application of DMBR in AD, which can be used to control the SRT and HRT during solid waste energy recovery (Cayetano et al., 2019). The membrane flux, effluent turbidity, and TMP between subsequent cleaning events from days 284–294 are shown in Fig. 3. The flux reached a stable performance after four efflux events. Then, the average fluxes and effluent turbidity turned to be stable after DM layer recovery (Fig. 3). Under the condition of periodic On/Off of the suction pump, TMP jumped to the upper bound (40 kPa) and then gradually decreased in a regular way. However, there was also a gradual increase of the lower bound of TMP. When this lower bound increased to 11.8–15.9 kPa (Fig. 3), the DM module needed to be backwashed using biogas for the recovery of

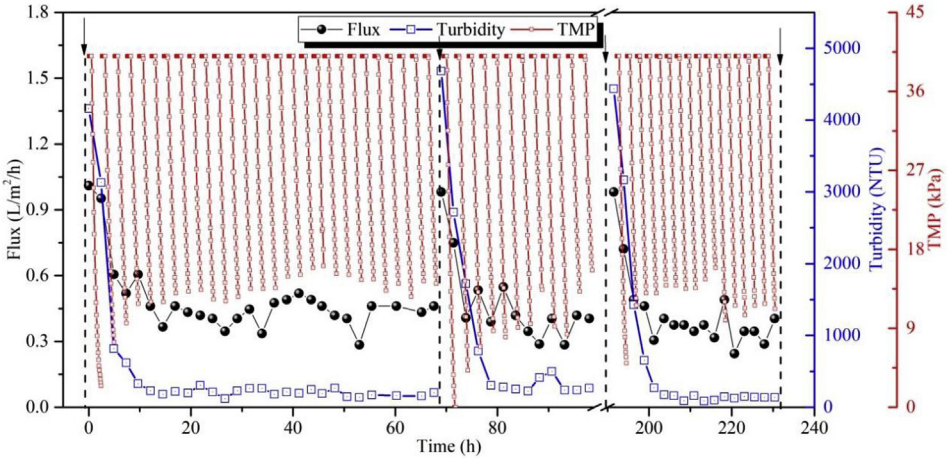

Fig. 3. Example of the evolution of membrane flux, effluent turbidity, and TMP between subsequent cleaning events (black vertical lines) from days 284–294.

filtration flux, following the authors' previous experiences (Hu et al., 2016; Tang et al., 2017). Pictures of the DM module with a stable filtering layer showed the DM layer to be compact and thin, as shown in Fig. S1. Meanwhile, compared with the morphology and SEM images of a new membrane and an air-dried fouled membrane without any pretreatment (Fig. S2), the nylon mesh was not deformed, which indicated that the nylon mesh was stable and durable for digestion in further practical application engineering. In addition, the PSD of reactor sludge being larger than those of feedstock and cake sludge at the end of Stage 10 (Fig. S3) indicated that the ability retained by the DM layer was efficient and beneficial for the further degradation of the substrate and may achieve higher conversion efficiency than continuous stirred tank reactors (CSTRs) under the same OLR and HRT.

3.2. High-rate performance of FW digestion with CM as an additive

3.2.1. High CH_4 production

As shown in Table 2 and Fig. 2, the average CH_4 production of 2.71 ± 0.32 L CH_4/L/day was achieved at 11.9 ± 0.87 g COD/L/day with effluent VFA levels of 4.1 ± 3.1 mg COD/L, which was 1.6–2.5 times of the reported CH_4 production of 1.1 L CH_4/L/day in treating high-strength FW using DMBR (Cayetano et al., 2019), 1.06–1.65 L CH_4/L/day in mesophilic FW fermentation using a CSTR-type reactor (Qiang et al., 2012, 2013). VFAs accumulation up to 1.2–3.0 g/L apparently hindered CH_4 production in these reported studies. VFAs accumulation at high OLRs would also bring about pH decrease which resulted in digester failure (Ma et al., 2018). CM addition to the DMBR in the current study thus significantly improved the condition for efficient fermentation. On the other hand, under an operation mode of continuous feeding of diluted FW, the CH_4 production could be as high as 2.78 L CH_4/L/day at 8.6 g COD/L/day with effluent VFA level controlled at 402 mg COD/L (Park et al., 2018). Thus, it can be inferred that changing the feeding mode from step-wise to continuous results in a higher CH_4 production rate and stable digestion of FW and CM in DMBR, which should be further investigated and confirmed in the future.

3.2.2. Reaction ratios and enzyme content

As shown in Fig. 4, the reaction ratios of the four AD steps (hydrolysis, acidogenesis, acetogenesis and methanogenesis) during the initial and steady period and lignocellulase and coenzyme F_{420} levels during the steady period were calculated and measured, respectively. Hydrolysis is generally the rate-limiting stage in AD of organic solid waste (Agyeman and Tao, 2014). As shown in Fig. 4(a), the hydrolysis rate increased significantly from $11.0 \pm 1.7\%$ to $28.1 \pm 1.4\%$ after 300 days of culture in the DMBR. Meanwhile, the acidogenesis, acetogenesis and methanogenesis rates were also increased (Fig. 4(a)), which was consistent with the improvement in CH_4 yield from 103 to 250 mL CH_4/g COD and high CH_4

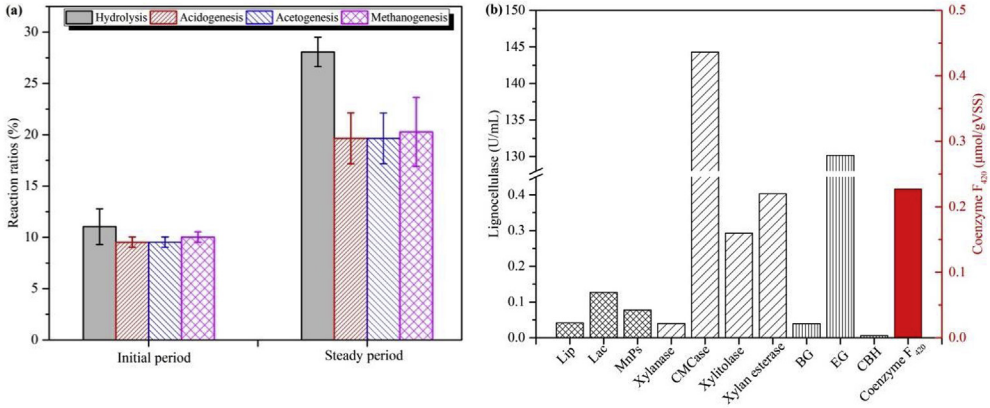

Fig. 4. Reaction ratios of the four anaerobic digestion steps using inoculum and digestion sludge from the DMBR during the steady period with the same organic loading of 7.1 ± 0.5 g VS/L using batch digestion tests (a), and lignin peroxidase (Lip), laccase (Lac), manganese peroxidase (MnPs), xylanase, carboxymethyl cellulase (CMCase), xylitolase, xylan esterase, β-glucosidase (BG), endoglucanase (EG), cellobiose hydrolase (CBH), and coenzyme F_{420} levels in DMBR during the steady period (b).

production (Table 2). The high CH_4 production correlated significantly with the coenzyme F_{420} activity of methanogens during anaerobic biodegradation. As shown in Fig. 4(b), the level of coenzyme F_{420} in DMBR was 0.23 μmoL/g VS during the steady period, which can be considered a rather high value with respect to those reported in the literature for AD systems (0.006–0.49 μmoL/g VSS) (Salkinoja-Salonen, 1982). Moreover, the lignocellulose-degrading enzyme is composed of lignin-degrading enzymes (Lip, Lac, and MnPs), hemicellulose-degrading enzymes (xylanase, CMCase, xylitolase, and xylan esterase), and cellulose-degrading enzymes (BG, EG, and CBH). The concentrations of lignocellulose-degrading enzyme in the DMBR were similar with the lignocellulose-degrading enzyme contents in cow rumen fluid (Xing et al., 2019), which was also consistent with the hydrolysis rate in the steady period (Fig. 4). These results suggest that the stable and high-rate digestion of FW and CM under an OLR of 11.9 ± 0.87 g COD/L/day was mainly due to high hydrolysis and methanogenesis rates, which had significant positive correlations with lignocellulase and coenzyme F_{420} concentrations. However, the hydrolysis rate was still the limiting rate for co-digestion with FW and CM. Therefore, improving the hydrolysis rate of lignocellulose biomass is still a key problem for further enhancing the bioenergy recovery efficiency of AD with FW and CM.

3.2.3. Degradation of cellulose, hemicellulose, and lignin

To definite the changes of lignocellulose of FW and CM in DMBR, the degradation of cellulose, hemicellulose, and lignin was investigated. Fig. 5 shows the XRD patterns and FTIR spectra of CM, FW, feedstock, and sludge samples from the DMBR digester at the end of Stage 10. The cellulose crystallinity was approximately 72.6% for feedstock and decreased to 16.4 ± 0.7% in the DMBR. This result indicated that 56.2% of the crystalline structure of cellulose in DMBR was removed, which was consistent with the 78.3% decrease in cellulose content (Table 3). The decreased crystallinity of

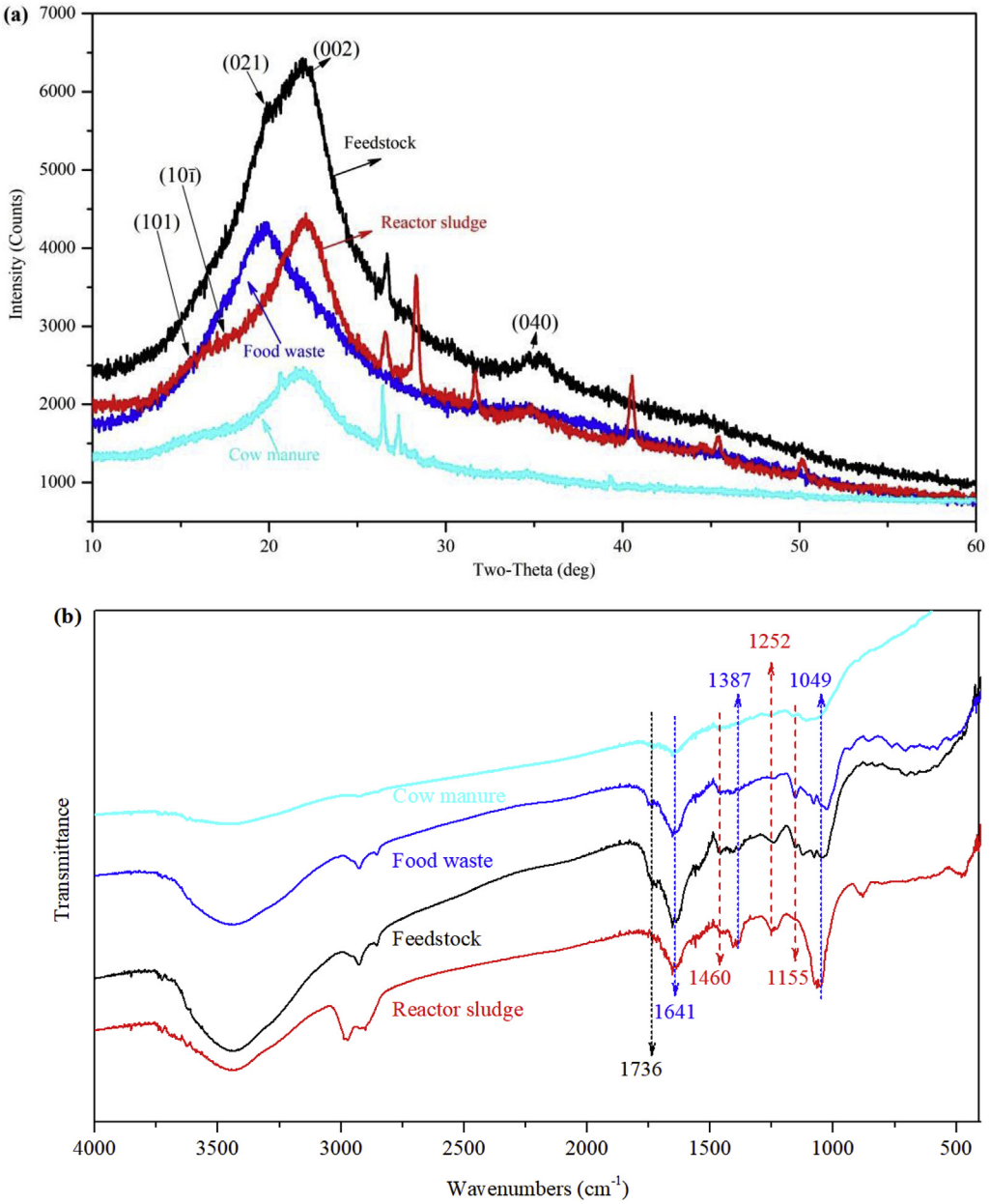

Fig. 5. X-ray diffraction (XRD) patterns (a) and FTIR spectra (b) of cow manure, FW, feedstock, and sludge sampling in the DMBR at the end of stage 8.

Table 3
Contents and removal efficiency of cellulose, hemicellulose, lignin, and TS of feedstock and sludge in the DMBR during the high-rate period with HRT of 10 days and SRT of 15.5 days.

Parameter	Cellulose	Hemicellulose	Lignin	TS
Feedstock contents	6.3 g/L (9.2%TS)	3.7 g/L (5.4%TS)	11.1 g/L (16.1%TS)	68.9 g/L
Reactor sludge contents	2.1 ± 0.2 g/L (5.5 ± 0.6%TS)	2.7 ± 0.1 g/L (6.9 ± 0.2%TS)	11.5 ± 0.8 g/L (29.8 ± 2.1%TS)	38.6 ± 2.3 g/L
Removal efficiency	78.3%	58.8%	47.5%	66.8%

cellulose might be attributed to the induced and selective enrichment of some cellulose-hydrolysing microbial species with CM as an additive, which will be further investigated in Section 3.3.

The FTIR spectra of the feedstock and sludge sampling in the DMBR are shown in Fig. 5(b). The relative absorbances of the characteristic bands of lignin at 1460 and 1155 cm^{-1} decreased, and those at 1252 cm^{-1} slightly increased with digestion, which indicated that the degradation of the aromatic methyl group and aromatic C—H in-plane deformation (guaiacyl type) was greater than that of the guaiacyl ring breathing with a C—O group. Meanwhile, the relative absorbances of the characteristic bands of cellulose at 1641 cm^{-1} decreased, and those at 1387 and 1049 cm^{-1} increased, which suggested that the bending of absorbed water decreased and was related to the main degradation component of cellulose. Additionally, the relative absorbances of the characteristic bands of hemicellulose at 1736 cm^{-1} decreased, which was consistent with the 58.8% decrease in hemicellulose content after digestion in the DMBR (Table 3).

As shown in Table 3, lignin content increased slightly from 11.1 g/L to 11.5 ± 0.8 g/L, and the lignin percentage increased from 16.1% to 29.8 ± 2.1%, which was consistent with the changes in FTIR spectra (Fig. 5(b)). Meanwhile, the cellulose level decreased significantly from 6.3 g/L to 2.1 ± 0.2 g/L, corresponding to a decrease in the cellulose percentage of 9.2% to 5.5 ± 0.6% during Stage 10 (HRT, 10 days; SRT, 15.5 days). The hemicellulose content also decreased from 3.7 g/L to 2.7 ± 0.1 g/L. Through complex and systematic calculations, the removal efficiencies of cellulose, hemicellulose, lignin, and TS were determined to be 78.3%, 58.8%, 47.5%, and 66.8%, respectively (Table 3), indicating that the amount of lignocellulose degraded in feedstock was in the order of cellulose > hemicellulose > lignin. The degradation efficiencies of the main components in feedstock can rival that of lignocellulosic waste using rumen microorganisms, as reported by Hu et al. (2008).

3.3. Analysis of the microbial community

3.3.1. Species diversity and richness

The high-rate and stable performance of FW fermentation with CM as additive has a closely relationship with the diversity and richness of microbial community in the DMBR system. The Shannon index and ACE/Chao 1 estimator are ecological indexes estimating species diversity and richness, respectively, as shown in Table 4. The coverage for bacteria, archaea, and fungi was more than 0.96, indicating that most sequences were detected. That the numerical values of these factors for the bacterial and archaeal sequences and OTUs were larger than those of fungi indicated that bacterial and archaeal diversity and richness greatly exceeded those of fungi (Table 4). The number of OTUs and the ecological indexes for bacteria and archaea of reactor sludge were higher than those of the seed sludge, which indicated that the addition of CM to FW digestion could increase the abundance and variety of bacteria and archaea. However, the reactor sludge had fewer OTUs and a lower Shannon index for fungi than for seed sludge, which meant that some of the fungal species faded away during the AD of FW and CM. Meanwhile, the ACE and Chao 1 estimator were higher for fungi than for seed sludge, which indicated that the addition of CM to FW could also increase the abundance of some fungi. An explanation for the increase in bacterial, archaeal, and fungal abundance in the reactor sludge could be that the mixture of FW and CM created complementary substrates, which in turn could stimulate bacterial, archaeal, and fungal growth and therefore enhance degradation and CH_4 production. The rich and diverse bacteria, archaea, and fungi in the FW and CM co-digestion system after long-term operation could thus provide microbiological evidence for the essential reasons of stable and high-rate CH_4 production achieved in the current study by DMBR operation as well as other related studies by different operation modes (El-Mashad and Zhang, 2010; Li et al., 2009; Marañón et al., 2012; Zhang et al., 2013) using CM as an additive.

3.3.2. Bacterial community

As shown in Fig. 6, the taxonomy of the OTUs of seed sludge, feedstock, and reactor sludge was classified at the genus level, and genera with a relative abundance higher than 1% were defined as the dominant genera. The dominant bacterial genera in reactor sludge were *Syntrophomonas* (13.32%), *Candidatus Cloacamonas* (13.27%), *Clostridium III* (4.41%), and *Sedimentibacter* (1.99%). The members of *Syntrophomonas*, as obligately anaerobic and syntrophic bacteria, have the ability to oxidize VFAs such as butyrate into acetate, which usually occurs in syntrophic association with a H_2-using methanogen and enriches those H_2-using methanogens (Zhang et al., 2017). The relative abundance (RA) of *Syntrophomonas* in seed sludge and feedstock were 0.03% and 0.01%, respectively. Their existence in the system, though not abundant in the start period, might have much induced functional microorganism growth and enhanced the digestion process. Other functional

Table 4
Richness and diversity indexes of bacterial, archaeal and fungal clone libraries.

	Specimens	Sequence number	OTUs	Shannon	ACE	Chao1	Coverage	Simpson
Bacteria	Seed sludge	73042	1721	2.288	67257	23015	0.979	0.226
	Feedstock	104466	3731	2.082	96122	34317	0.966	0.422
	Reactor sludge	112757	2817	3.296	136178	38264	0.976	0.101
Archaea	Seed sludge	154961	2324	0.627	410073	87338	0.985	0.781
	Feedstock	133840	3377	1.744	139152	45552	0.977	0.456
	Reactor sludge	124830	2787	1.376	677616	138739	0.978	0.493
Fungi	Seed sludge	55877	631	2.588	6120	2610	0.992	0.222
	Feedstock	55227	541	1.239	5085	3180	0.993	0.431
	Reactor sludge	49935	404	2.166	7132	3740	0.994	0.336

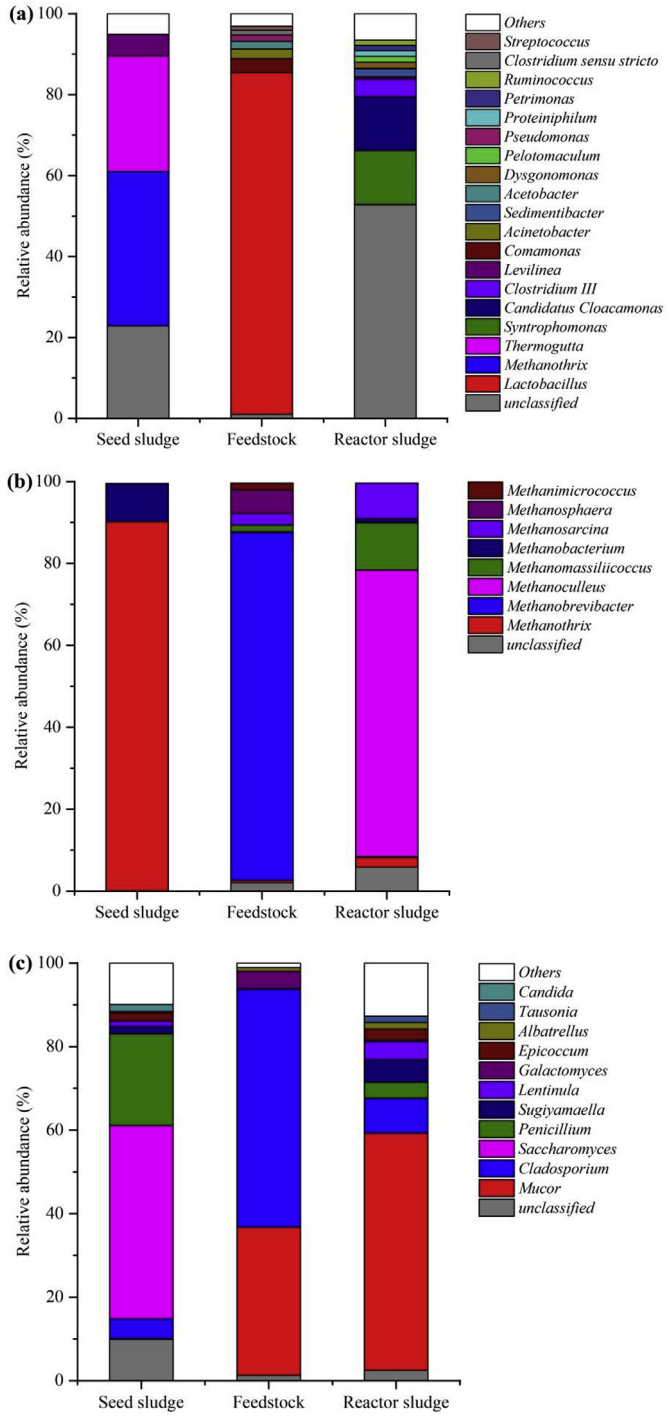

Fig. 6. Similarity and relative abundance variation of bacteria (a), archaea (b), and fungi (c) of feedstock, inoculum, and reactor sludge on day 262.

microorganisms, such as *Candidatus Cloacamonas*, *Clostridium III*, and *Sedimentibacter*, were not from the seed sludge but from CM. Although their RA values were very low in the feedstock, they also performed important role to enhance the digestion process. Members of *Candidatus Cloacamonas*, branching from the *Spirochaetes*, were previously found to be hydrogen-producing syntrophs that are involved in the oxidation of propionate into acetate and CO_2 (Pelletier et al., 2008). This reaction is thermodynamically favourable only when the H_2 partial pressure remains low and occurs by coupling the propionate-oxidizing reaction with the hydrogen-utilizing reaction mediated by hydrogenotrophic methanogens (Li et al., 2017b). Therefore, the dominance of *Candidatus Cloacamonas* might be attributed to the promoted growth of hydrogenotrophic methanogens of *Methanomassiliicoccus* in the DMBR, as shown in Fig. 6(b). *Clostridium III* was a genus of bacteria in the family *Ruminococcaceae*, whose members are able to hydrolyse a diverse range of lignocellulosic biomass (Li et al., 2018b). *Sedimentibacter* species are amino acid-utilizing bacteria that degrade the amino acids and produce acetate, propionate, ammonia, etc. as end-products (Imachi et al., 2016). Ammonia ensures sufficient buffer capacity of AD and maintains a neutral pH, thus increasing the stability of AD (Zhang et al., 2017), which is a possible main reason for the high CH_4 production in the DMBR. Obviously, using CM as an additive is worth the in situ bioaugmentation in the AD of FW.

The most abundant bacterial genus in feedstock was *Lactobacillus*, which belongs to the *Lactobacillaceae* family. The abundance of *Lactobacillus* dropped as the pH increased from 4.0 to 5.0, as reported by Tang et al. (2017), which is in accord with the lower abundance of *Lactobacillus* in the reactor sludge (0.05%) with a pH of 8.39 than in the feedstock (84.39%). Moreover, many genera were washed out from the seed sludge after long-term AD, e.g., *Methanothrix*, *Thermogutta*, and *Levilinea*. Additionally, the RA of unclassified in seed sludge, feedstock, and reactor sludge was 22.88%, 1.03%, and 52.77%, respectively. Explicitly identifying the species and metabolic pathways of those unclassified bacteria using metagenomic approaches will be beneficial to further explaining the high CH_4 production and stability of FW digestion with CM as an additive that was observed in this study.

3.3.3. Archaeal community

Fig. 6(b) shows the methanogenic microbial composition at the genus level. The majority of sequence reads were assigned to *Methanoculleus*, *Methanomassiliicoccus*, *Methanosarcina*, *Methanothrix*, and *Methanobacterium*, and the five genera accounted for 70.01%, 11.45%, 8.74%, 2.41%, and 1.03% of the total methanogenic microbial composition, respectively. Therefore, the total proportion of hydrogenotrophic methanogens (*Methanoculleus*, *Methanomassiliicoccus*, and *Methanobacterium*) was higher than that of acetoclastic methanogens (*Methanothrix*). Many researchers have indicated that acetoclastic methanogens are more sensitive to ammonium inhibition than hydrogenotrophic methanogens (Xie et al., 2014; Yang et al., 2019). Therefore, the acetate-utilizing methanogens were inhibited in the presence the high NH_4^+-N concentrations (1880 mg/L) mentioned above, which was consistent with the RAs of *Methanothrix* in seed sludge and feedstock of 90.14% and 0.52%, respectively. Moreover, *Methanosarcina* is the most metabolically and physiologically versatile methanogen that can convert different substrates, such as acetate, H_2, and methyl containing groups to CH_4 (Kurade et al., 2019; Zhang et al., 2017). The enrichment of *Methanosarcina* might have enhanced CH_4 production by using various substrates, which was in agreement with the results of CH_4 production (Fig. 2). Similarly, the major archaea in the reactor sludge, including *Methanoculleus* and *Methanomassiliicoccus*, were also not detected in seed sludge, indicating that the CM addition enhanced the diversity of the archaeal community during the FW and CM digestion process.

Five archaeal genera, i.e., *Methanobrevibacter* (85.07%), *Methanosphaera* (5.73%), *Methanosarcina* (2.78%), *Methanimicrococcus* (1.7%), and *Methanomassiliicoccus* (1.58%) were dominant in the feedstock, accounting for more than 96.86% of the total archaeal population. Potential aciduric methanogens such as *Methanobrevibacter* and *Methanomassiliicoccus* were responsible for biomethanation from hemicellulose (Li et al., 2018b). These characteristic bacteria and methanogens are worth using for in situ

bioaugmentation in the AD of lignocellulosic biomass.

3.3.4. Fungal community

Prevalent fungal genera detected in the seed sludge, feedstock, and reactor sludge are shown in Fig. 6(c). The identified fungi in reactor sludge mainly fell into five genera: *Mucor* (56.77%), *Cladosporium* (8.32), *Sugiyamaella* (5.43%), *Lentinula* (4.36%), and *Penicillium* (3.74%). The most abundant fungal species in reactor sludge was *Mucor circinelloides* (56.55%). *M. circinelloides* can secrete a CMCase and cellulase for degrading crystalline cellulose (Baba et al., 2005) and is one of the dominant fungal species in the feedstock (35.43%). *Cladosporium*, a lignin- and cellulose-degrading fungal strain (Jin et al., 2012), secretes Lac and EG and is found mostly in feedstocks, with a RA of 59.66%. *Sugiyamaella xylanicola* (5.41%) is a xylan-degrading species. As reported by Morais et al. (2013), *S. xylanicola* is able to grow in medium with xylan as sole carbon source and produces extracellular enzymes with xylanolytic activities. Moreover, *Lentinula* and *Penicillium* contain species that degrade lignin and cellulose (Ohga and Royse, 2001; Sun et al., 2015). The *Saccharomyces* genus was washed out in the reactor sludge due to a high pH of 8.0, which is not suitable for its multiplication.

Fungi produce many different lignocellulolytic enzymes (Fig. 4(b)) that are considerably important for the biodegradation of lignocellulose. Meanwhile, the mycelia of fungi can penetrate cellulose to create pores, thus increasing the available surface area for enzymatic attacks and correspondingly increasing the hydrophilicity of cellulose (Chen et al., 2017; Hu et al., 2008). Furthermore, fungal groups can produce a large amount of H_2 and CO_2 while decomposing cellulose, which is suitable for the metabolism of hydrogenotrophic methanogens in the DMBR. The association of methanogens and fungi can be further attributed to cellulose degradation and result in the significant acceleration of cellulose degradation (Chen et al., 2017). To develop an in-depth understanding of the relationship between bacteria, archaea, and fungi, the main metabolic pathways of the microbial communities were derived from measurements of end product fluxes in combination with reviews of the published literature (Fig. 7). The cellulose, hemicellulose, and lignin of feedstock were mainly degraded by fungi (the genera *M. circinelloides*, *S. xylanicola*, *Lentinula*, and *Penicillium*) and bacteria (the genus *Clostridium III*). The fermentation products (pentose and hexose sugar) can be converted to pyruvate and then produced VFAs. Meanwhile, the other products (H_2 and CO_2) can be utilized by hydrogenotrophic methanogens (*Methanoculleus*, *Methanomassiliicoccus*, and *Methanobacterium*) with a high tolerance of ammonium inhibition. Thus, the biodegradation of lignocellulose further improved the CH_4 yield of FW digestion with CM as additive. On the other hand, the protein in DMBR was mainly degraded by the genera *Sedimentibacter*, and the produced NH_4^+-N further improved the AD buffer capacity. A virtuous cycle was formed between bacteria, archaea, and fungi in the stable and high-rate DMBR reactor. Therefore, using CM as additive is a promising alternative for improving stability and biogas production of FW digestion due to bioaugmentation with bacteria, archaea, and fungi.

4. Conclusions

A long-term, stable and high-rate anaerobic mesophilic digestion of FW was achieved with CM as an additive in a semi-continuous DMBR. Adding CM to the FW digestion resulted in a better C/N balance, increased buffer capacity, and the stable DM module, which were beneficial for the stability of the anaerobic process. The average CH_4 yield and CH_4 production were 250 mL/g COD and 2.71 L CH_4/L/day, respectively, at an OLR of 11.9 g COD/L/day, HRT of 10 days, and SRT of 15.5 days. The degradation rates of cellulose, hemicellulose, and lignin were 78.3%, 58.8%, and 47.5%, respectively, as could be verified by the XRD and FTIR spectra. The improved reaction ratios, coenzyme F_{420} content, and lignocellulose-degrading enzyme levels were significantly

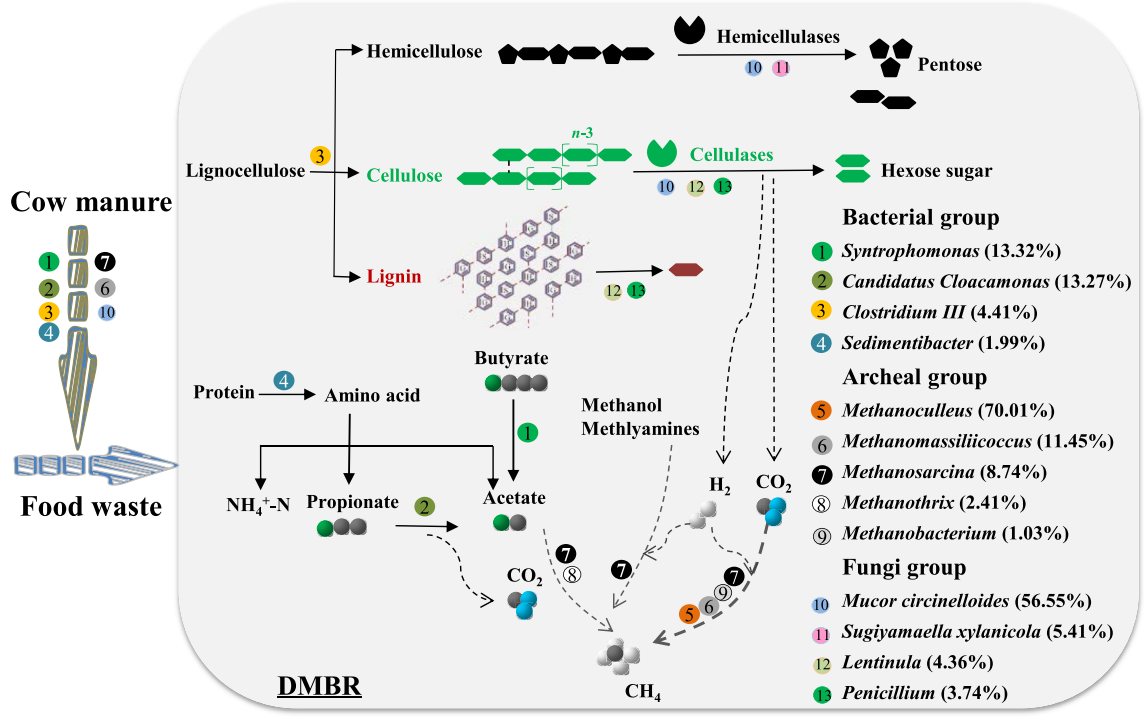

Fig. 7. A schematic showing the major metabolic pathways of the bacteria, archaea, and fungi during FW digestion with CM as an additive in this study.

correlated with the high-rate performance of FW and CM digestion and the microbial community in DMBR sludge. The abundance and number of varieties of bacteria, archaea, and fungi in the DMBR reactor were determined to be enhanced with CM addition through high-throughput sequencing analysis. The biodegradation of lignocellulose can further improve the CH_4 yield of FW digestion and produce H_2 and CO_2 for hydrogenotrophic methanogens with a high tolerance of ammonium inhibition, and ammonium then improves the AD buffer capacity. A virtuous cycle was formed in the FW digestion system with CM as an additive that was also responsible for the stable and high-rate CH_4 production by FW digestion. Further studies may still be required for optimizing the scheme of CM addition to achieve high rate FW digestion without large quantity of additive material and extra operation cost.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.115099.

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