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Symbiotic *Bacteroides* and *Clostridium*-rich methanogenic consortium enhanced biogas production of high-solid anaerobic digestion systems

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ABSTRACT

The high-solid anaerobic digestion (AD) for anaerobic co-digestion of palm oil mill effluent (POME) and oil palm empty fruit bunches (EFB) has low degradation efficiency. The enhancement of biogas production and EFB degradation efficiency by *Bacteroides* and *Clostridium*-rich methanogenic consortium was investigated. A *Bacteroides* and *Clostridium*-rich methanogenic consortium have xylanase, FPase, *endo*-glucanase, and *exo*-glucanase activity of 9.2, 1.3, 2.1, and 1.5 IU·mL^{-1}, respectively with EFB degradation efficiency of 57.5%. Methane production (79 m^3-CH_4·tonne^{-1}-EFB) by *Bacteroides* and *Clostridium*-rich methanogenic consortium augmentation was increased by 67.2%. The real application of bioaugmentation in the feedstock mixing tank before a feed to the AD reactor significantly increased methane yield (376.7 mL-CH_4·g^{-1}-VS^-) by 85.5%. Bioaugmentation of *Bacteroides* and *Clostridium*-rich methanogenic consortium increased the number of hydrolytic bacteria and strengthened symbiotic relationships with indigenous microorganisms resulting in high biogas production, accelerated hydrolysis, and reduced solids accumulation of the high-solid AD process.

1. Introduction

The recalcitrance structure of cellulose, hemicellulose, and lignin is usually considered a rate-limiting step in the anaerobic digestion (AD) process (O'Sullivan and Burrell, 2007). Thus, the proper pretreatment or other enhancement strategies are integrated into the AD process for enhancing the solubility of lignocellulosic biomass (Tomei et al., 2009). Adding hydrolytic and hemicellulolytic microorganisms to improve feedstock degradation and biogas production is widespread and reasonable (Deng et al., 2017; Kong et al., 2018; Suksong et al., 2019). Hydrolytic and hemicellulolytic microorganisms can degrade lignocellulosic biomass by associated with non-cellulolytic microorganisms, which enhance the synthesis of essential nutrients that promote cellulose degradation (Haruta et al., 2002). *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Chloroflexi* were predominated in anaerobic digesters based on a sequence-based meta-analysis observed in the AD systems (Nelson et al., 2011). Metatranscriptomic characterization in methane production from cellulose demonstrated that *Thermotogales*, *Clostridiales*, and *Bacteroidetes* involving in cellulose degradation (Xia et al., 2014). Campanaro et al. (2016) found that the phyla *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* were dominated in the AD sludge by metagenomics analysis and were involved in the degrading of polysaccharides. High biodegradation of crop residues under the AD was frequently related to the abundance of *Bacteroidetes* (Qiao et al., 2013; Yan et al., 2012). The enrichment of a microbial consortium was effectively degraded switchgrass comprising *Firmicutes*, *Bacteroidetes*, and *Alphaproteobacteria* (DeAngelis et al., 2012). Anaerobic bacteria degrade lignocellulosic biomass in various environments involving *Clostridium* and *Bacteroides* (Wright and Klieve, 2011). Weiß et al. (2010) enhanced biogas production from agricultural biomass by 53% with the addition

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of hemicellulolytic consortium highly dominated by *Bacteroides* sp., *Dechlorosoma* sp., and *Clostridium* sp., while co-culture of *Bacteroides cellulosolvens* and *Clostridium saccharolyticum* showed increasing cellulose degradation of 33% compared to *B. cellulosolvens* alone (Murray, 1986). Haruta et al. (2002) constructed a high cellulose degradation microbial consortium, dominated by the coexistence of *Clostridium thermosuccinogenes* and *Pseudoxanthomonas taiwanensis* for rice straw degradation, with an efficiency of 60% within 4 days. Most reported microbial consortia were used for prehydrolysis rather than combined degradation with methanogenesis, resulting in extra investment expenses for equipment and energy consumption. Various microorganisms can perform biodegradation of lignocellulose and a balanced enzyme complement has been observed in a multispecies consortium (Zhu et al., 2016).

Recently, a mixture of bacteria and archaea augmented into the AD process to treat sewage sludge, improved biodegradation efficiency by more than 46% (Lebiocka et al., 2018). Bioaugmentation increased synergistic activity between exogenous microorganisms and indigenous AD microorganisms, leading to enhanced biogas yield (Weiß et al., 2010). Bioaugmentation of methanogenic sludge and cellulolytic rumen bacteria increased methane yields from rice straw for 2.3-fold compared to methanogenic sludge alone, while also increasing the proportion of cellulolytic *Clostridium* and *Ruminococcus* (Deng et al., 2017). Ozbayram et al. (2017) augmented *Ruminococcaceae* and *Lachnospiraceae* rich methanogenic consortium into an AD reactor and increased methane production by 27% from wheat straw. Enrichment of cellulolytic bacteria on different plant biomass along successive transfers favored archaea, with the concomitant production of methane (Sträuber et al., 2015). However, in order to boost the degradation efficiency of the lignocellulosic biomass, it has never been mentioned that a *Bacteroides* and *Clostridium*-rich methanogénic consortium were used.

Here, the potential of a *Bacteroides* and *Clostridium*-rich methanogenic consortium was investigated to enhance biodegradability and biogas production from the high-solid AD of EFB. The 16S rRNA sequencing approaches were investigated to assess the microbial community of *Bacteroides* and *Clostridium*-rich methanogenic consortium and responses of the indigenous microbial community from bioaugmentation.

2. Materials and methods

2.1. Feedstock and methane-producing sludge

Methanogenic sludge inoculum, oil palm empty fruit bunches (EFB), and palm oil mill effluent (POME) were obtained from Nam Hong Palm Oil Co., Ltd., Krabi, Thailand. EFB was dried under 95 °C for 2 days to remove the moisture and ground to 5 mm. POME was stored at 4 °C until used as substrate. The characteristics of EFB and POME are shown in Table 1. The methanogenic sludge inoculum with volatile suspended solids (VSS) of 48.3 g L^{-1} was used as inoculum.

Table 1
Characteristics of palm oil mill effluent and oil palm empty fruit bunches used in this study.

Characteristics	POME	EFB	Unit
Moisture content	94.85	77.45	%wet weight
Total solids (TS)	5.15	26.55	%wet weight
Volatile solids (VS)	4.27	20.11	%wet weight
Ash	0.88	6.44	%wet weight
VS/TS	0.83	0.89	%wet weight
Total nitrogen (TN)	0.06	0.16	%wet weight
Chemical oxygen demand (COD)	64.95	216.7	$g{\cdot}kg^{-1}$
C: N ratio	27	64	–
Cellulose	–	41.21	% of TS
Hemicellulose	–	26.32	% of TS
Lignin	–	20.00	% of TS
Lipids content	0.71	0.26	%wet weight
pH	4.48	7.02	–

2.2. Enrichment of Bacteroides and Clostridium-rich methanogenic consortium

Bacteroides and *Clostridium*-rich methanogenic consortium were enriched from dairy cattle dung (CD), horse dung (HD), and soil sediment (SD), biogas effluent (EF), and 45 days of EFB compost according to the procedure previously described by Weiß et al. (2010). Ten grams of CD, HD, SD, EF, and 45DC samples were homogenously mixed in 90 mL of the basal anaerobic medium containing 5 $g{\cdot}L^{-1}$ xylan and microcrystalline cellulose as the carbon source in a 125 mL serum bottle. A filter paper strip was used to determine the lignocellulolytic enzyme activity (Wongwilaiwalin et al., 2010). All enriched experiments were conducted in triplicates. The bottle headspace was flushed with nitrogen to create anaerobic conditions and the bottles were covered with rubber stoppers, sealed with aluminum caps, and incubated at 35 °C for 7 days. The enriched cultures were routinely transferred to a new medium every 7 days and analyzed for cellulase (CMCase, Avicelase, and FPase) and xylanase activities. In the enriched cultures, dominant microorganisms were confirmed by nested polymerase chain reaction-denaturing gradient gel electrophoresis (nested PCR-DGGE). Cellulolytic and methanogenic properties were confirmed by quantitative real-time PCR (qPCR) targeting the glycoside hydrolase family 6 cellulase (*GH6*) and the methyl coenzyme M reductase A (*mcrA*) as a functional gene.

2.3. Hydrolysis of EFB by Bacteroides and Clostridium-rich methanogenic consortium

The enriched *Bacteroides* and *Clostridium*-rich methanogenic consortium was tested for their ability to degrading EFB under anaerobic conditions following the method of Wang et al. (2011). Briefly, 10 mL of each enriched culture ($OD_{600nm} = 0.582$) were inoculated into 90 mL of basal anaerobic medium broth supplemented with 1% of EFB (based on volatile solids). All hydrolysis experiments were statically incubated at 35 °C for 7 days. The whole culture broth was filtered with a 0.45 μ syringe filter to recover the remaining EFB. The solid fraction was mixed with a 20 mL acetic acid/nitric acid reagent to remove the bacterial cells. The mixture was heated for 30 min at 100 °C. The heated mixture was filtered and washed with 20 mL distilled water 3 times. Subsequently, the remaining EFB was dried at 90 °C for 2 days (Feng et al., 2011). The fermentation broth was analyzed for acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid, and valeric acid by gas chromatography connected to a flame ionization detector (GC-FID). Degradation efficiency was determined based on dry weight using the following equation (Eq. (1)) and EFB hydrolysis was confirmed by scanning electron microscope examination of surface area to determine the porosity.

$$\text{Degradation efficiency}\,(\%) = [(\text{added EFB} - \text{remaining EFB})/\text{added EFB}] \times 100 \quad (1)$$

2.4. Augmentation of Bacteroides and Clostridium-rich methanogenic consortium in the AD process

Methane production from EFB with *Bacteroides* and *Clostridium*-rich methanogenic consortium augmentation was conducted by batch fermentation. Ten percentage (*v*/v) of enriched *Bacteroides* and *Clostridium*-rich methanogenic consortium augmentation (CD, HD, SD, EF, and 45DC) with excellent EFB degradation performance were augmented into the AD process. The EFB and methanogenic inoculum based on volatile solids (VS) ratio was 2:1 at a total solid (TS) of 10%. The EFB and methanogenic inoculum were mixed with *Bacteroides* and *Clostridium*-rich methanogenic consortium and fed into the 500 mL serum bottles at a working volume of 300 mL. Bottle headspace was purged with nitrogen gas to remove oxygen from the system and covered

with rubber stoppers and aluminum caps. The serum bottles were incubated at 35 °C for 50 days. The negative control consisted of methanogenic sludge inoculum and *Bacteroides* and *Clostridium*-rich methanogenic consortium without substrate. Biogas data analysis has been conducted using SPSS Statistics version 17.0 to identify any statistically relevant variations in methane production.

2.5. Augmentation of Bacteroides and Clostridium-rich methanogenic consortium in AD process simulation

The best *Bacteroides* and *Clostridium*-rich methanogenic consortium (CD consortium) with high methane production and EFB degradation efficiency was tested in the AD reactor. Simulation of actual application *Bacteroides* and *Clostridium*-rich methanogenic consortium for biogas production was carried out in 1 L glass bioreactors. Simulation of applied CD-consortium for biogas production from anaerobic co-digestion of POME and EFB followed a similar method to real biogas plants, with separate hydrolysis by CD-consortium before feeding to AD reactor (TR1), separated hydrolysis by CD-consortium with the addition of easy degradable POME before feeding to the AD reactor (TR2), direct augmentation CD-consortium into AD reactor (TR3) and mixed CD-consortium with feedstock before feeding to AD reactor (TR4). In the TR1, EFB was pre-hydrolyzed with the CD-consortium for 7 days and the whole mixture was fed into the AD digester. In the TR2, EFB was mixed with 10% POME and pre-hydrolyzed with CD-consortium for 7 days. The prehydrolyzed POME and EFB was fed into the AD digester. In the TR3, CD-consortium was directly augmented into AD digester. In the TR4, CD-consortium was mixed with the substrate in the mixing tank before feeding into the AD digester. The reactors were started with methane-producing inoculum to substrate ratio based on a VS of 2:1 with TS content of 10% in all reactors. The reactors temperature was maintenance at 35 °C for 50 days. The volume and composition biogas were daily measured by the gas meter and the gas chromatography. Triplicates liquid and biogas samples were collected from the reactors for chemical parameters and gas composition analysis.

2.6. Analytical methods

Compositions of EFB, POME, and methane inoculum were determined by standard methods (APHA, 2012), while lignin, cellulose, and hemicellulose in EFB were calculated according to the procedure of Van Soest et al. (1991). The CHON compositions of the substrate, biogas volume, and biogas composition were analyzed as previously described by Suksong et al. (2019). The following equation (Eq. (2)) has been used to convert methane volume at 35 °C to standard temperature and pressure (STP) conditions.

$$\frac{V_1}{V_2} = \frac{T_1}{T_2} \tag{2}$$

where V_1, V_2, T_1, and T_2 were methane volume at 35 °C, methane volume at STP (22.4 L), incubation temperature (273.15 + 35 K), and temperature of STP (273.15 K), respectively. Gas chromatography connected to a Stabilwax®-DA (fused silica 30 m column) was used for volatile fatty acids analysis as previously described by Suksong et al. (2019). Xylanase and Cellulase (CMCase, Avicelase, and FPase) activities were assayed according to Nitisinprasert and Temmes (1991). The international enzyme unit (IU) is defined as the amount of enzyme releasing one μmol of reducing sugar per minute under test conditions.

Nested PCR-DGGE was used to analyze the microbial population, as previously described by Prasertsan et al. (2017). Microbial community profiles of the CD-consortium, the CD-consortium augmented AD system, and the unaugmented AD system was determined by next-generation sequencing on an Illumina MiSeq device previously described by Suksong et al. (2019). Quantitative real-time PCR (qPCR) was applied to determine cellulolytic and methanogenic communities of *Bacteroides* and *Clostridium*-rich methanogenic consortium by targeting the glycoside hydrolase family 6 cellulase (*GH6*) and the methyl coenzyme M reductase A (*mcrA*) functional genes according to the procedure described by Franke-Whittle et al. (2014). The purified PCR products of *Cellulomonas* sp. HD19AZ1 NBRC 113345 and *Methanosarcina barkeri* DSMZ 800 were used as standard DNA. The primer pairs of cell2F/cell2R (Merlin et al., 2014) and mlas-F/mcrA-R (Steinberg and Regan, 2009) were used for the *GH6* and the *mcrA* gene, respectively. Stock concentration (gene copies·μL^{-1}) of the standard DNA was determined via QuantiFluor® dsDNA Dye measurement for standard curve construction with ten-fold dilutions ranging from 10^9 to 10^1 copies·μL^{-1}. The Gompertz equation (Eq. (3)) was employed to evaluate the methane production as previously described by Mamimin et al. (2019). The Buswell equation (Eq. (4)) and first-order kinetic model (Eq. (5)) was used to estimate the theoretical methane yields and the hydrolysis constants (k_h), respectively, as previously described by Mamimin et al. (2019).

$$Y(t) = Y_{max} \times exp\left(-exp\left(\frac{R_{max} \times e}{Y_{max}} \times (\lambda - t) + 1\right)\right) \tag{3}$$

$$C_aH_bO_cN_d + \left(\frac{4a - b - 2c + 3d}{4}\right)H_2O \rightarrow \left(\frac{4a + b - 2c - 3d}{8}\right)CH_4 + a - \left(\frac{4a + b - 2c - 3d}{8}\right)CO_2 + d\ NH_3 \tag{4}$$

$$ln = \frac{B_0}{B_0 - B_t}\ k_h t \tag{5}$$

Balance in the generation and consumption of energy to ascertain the economic profitability of the bioaugmentation reactor employed in this study was evaluated. In doing this, a comparison was made between the extra expenses incurred (medium for microbial cultivation, agitation, and pump) and the extra energy obtained through the application of bioaugmentation. The evaluation was to see if the additional gas yield will suffice for the initial expenses.

3. Results and discussion

3.1. Enrichment of Bacteroides and Clostridium-rich methanogenic consortium

The enriched *Bacteroides* and *Clostridium*-rich methanogenic consortium CD, HD, EF, 45DC, SD, and EF (cattle dung, horse dung, biogas sludge, 45 days fermented compost, soil sediment, and biogas effluent) were completely degraded microcrystalline cellulose within 5 days. The enriched consortium has *endo*-β-glucanase, *exo*-β-glucanase, FPase, and xylanase activities ranging from 0.3–1.5, 0.7–2.1, 0.2–1.3, and 2.3–9.2 IU·mL^{-1}, respectively (Table 2). The CD-consortium showed the highest lignocellulolytic enzyme activity, followed by SD, HD, 45DC, and EF consortium, respectively. All enriched consortium has high xylanase and FPase activities. A high xylanase activity of 18.2 IU·mL^{-1} was observed in a microbial consortium enriched for rice straw degradation (Zhang et al., 2018). Biodegradation of cellulose and hemicellulose in the AD

Table 2
The characterization of the five enriched microbial consortia (MC). Values are means ± standard error.

MC	Enzyme activity (IU·mL^{-1})				Functional gene (gene copies·g^{-1} pellet)		Dominated bacteria
	Avicelase	CMCase	FPase	Xylanase	*mcrA* gene (×10^8)	*GH6* gene (×10^8)	
CD	1.5 ± 0.01	2.1 ± 0.05	1.3 ± 0.02	9.2 ± 0.08	8.9	1.2	*Bacteroides* sp. *Clostridium* sp.
HD	0.9 ± 0.01	1.2 ± 0.04	0.9 ± 0.01	2.9 ± 0.03	5.8	0.6	*Clostridium* sp. *Bacteroides* sp.
EF	0.3 ± 0.02	0.7 ± 0.01	0.7 ± 0.01	2.3 ± 0.04	3.1	0.9	*Clostridium* sp. *Moryella* sp.
SD	0.7 ± 0.01	1.1 ± 0.05	0.9 ± 0.01	6.3 ± 0.05	1.9	1.1	*Clostridium* sp. *Bacteroides* sp.
45DC	1.0 ± 0.01	0.8 ± 0.01	0.2 ± 0.01	3.5 ± 0.01	1.2	1.2	*Clostridium* sp. *Cellulosilyticum* sp.

systems was involved with exo-β-glucanase, endo-β-glucanase, cellulase, and xylanase activity (Schwarz, 2001). The enriched consortium had high copy numbers of gene *GH6* and the *mcrA* gene, indicating the activity of lignocellulolytic bacteria and methanogenic archaea. Glycoside hydrolases (*GH*) are enzymes that break down the glycosidic bond between a carbohydrate and a noncarbohydrate moiety and between two or more carbohydrates. The *GH6* family contains several cellulolytic enzymes, including cellobiohydrolase (EC 3.2.1.91) and endoglucanase (EC 3.2.1.4) (The CAZypedia Consortium, 2018). The *mcrA* gene is unique to the methanogens and presents principally congruent phylogeny to the 16S rRNA gene (Steinberg and Regan, 2008). Methane content in the gas phase of the enriched consortium was 12–26% at the end of the batch transfer, indicating that the enrichment procedure enriched both lignocellulolytic bacteria and methanogens.

The enriched consortium was dominated by the phyla *Firmicutes* and *Bacteroidetes*, which are well-known as effective lignocellulose degraders (Fig. 1). *Clostridium* sp. and *Bacteroides* sp. were predominant in CD, HD, and SD consortium while EF and 45DC consortia were dominated by *Clostridium* sp., *Moryella* sp., and *Cellulosilyticum* sp. The microbial community of the CD consortium was primarily composed of *Bacteroides*, *Clostridium*, *Acetoanaerobium*, and *Sarcina*. Cultivation of anaerobic sludge using xylan as the sole carbon source enriched species belonging to the genera *Clostridium*, *Bacillus*, *Bacteroides*, and *Pseudomonas* (Klocke et al., 2007). The microbial consortium enriched from the soil for rice straw degradation was dominated by *Clostridium* with high xylanase activity and efficient lignocellulosic biomass degradation (Zhang et al., 2018). *Bacteroides* sp. is a well-known decomposer of biomass, whereas *B. succinogenes* and *B. ruminicola* are among the main cellulolytic bacteria in the rumen with the ability to produce *endo*-β-glucanase, cellulase, and xylanase enzymes (Weiß et al., 2010). *Clostridium* sp. is a common bacterium for the degradation of hemicellulose and cellulose (Schwarz, 2001). *Clostridia butyricum* and *C. beijerinckii* have significant hydrolyzing activity (Wang et al., 2007). The archaeal community in the enriched consortium was dominated by *Methanosaeta*, *Methanobacterium*, *Methanosarcina*, and *Methanomasiliicoccus*. *Methanosaeta* and *Methanosarcina* are aceticlastic methanogens that use acetate in methanogenesis pathways (Welte and Deppenmeier, 2014). *Methanomasiliicoccus* produced methane by reducing hydrogen and methanol as electron donors (Dridi et al., 2012). The biological activity of methanogens, such as acetate reduction to methane, requires hydrogen to carry out redox reactions. Therefore, it is syntrophically linked to acetogenic species via the inter-species transfer of hydrogen (Fang et al., 2002). These results indicated that all cellulolytic bacteria were synergistic with methanogens that could consume VFAs and reduce their toxicity and maintain the pH in enriched cultures.

3.2. Hydrolysis of EFB by Bacteroides and Clostridium-rich methanogenic consortium

The enriched consortium CD, HD, EF, SD and 45DC had EFB degradation efficiency of 57.5%, 48.9%, 51.1%, 55.1% and 45.2%, respectively (Table 3). The CD consortium exhibited the highest EFB degradation efficiency within 7 days, followed by SD, EF, HD, and 45DC, respectively. The main soluble metabolites of the enriched consortium were acetic acid, butyric acid, and propionic acid. Butyric acid, acetic acid, and propionic acid were found to dominate in the lignocellulolytic microbial consortia fermentation system previously reported by Tuesorn et al. (2013). The microbial consortium dominated by *Bacteroides* and *Clostridium* was efficient in hydrolyzing corn stalk and enhance biogas production with a degradation efficiency of 45% (Xufeng et al., 2011). *Bacteroides* and *Clostridium* species contribute to the bioconversion of renewable raw materials into biogas through the degradation of plant

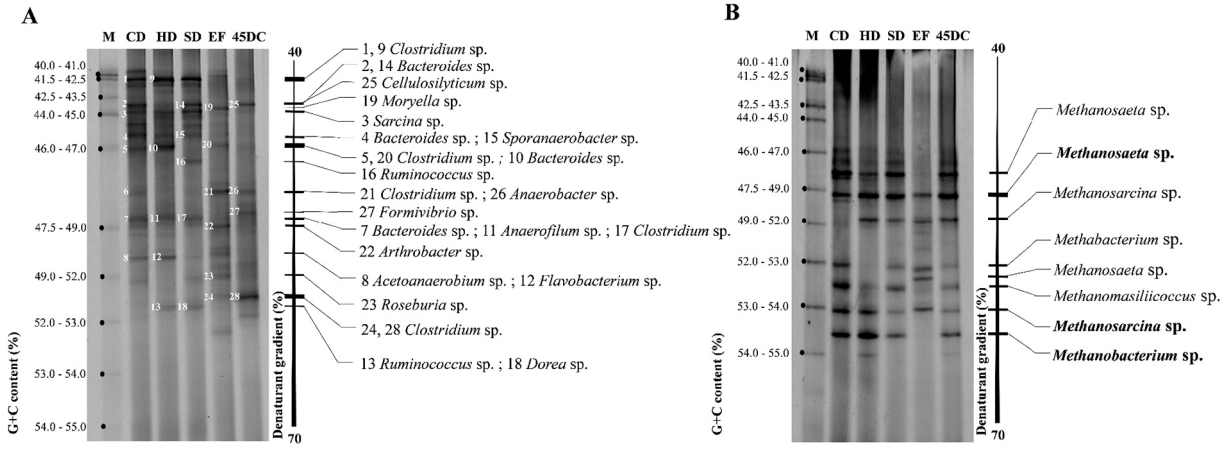

Fig. 1. Diversity and dominant bacteria (A) and archaea (B) in each enriched culture by denaturing gradient gel electrophoresis. M, CD, HD, SD, EF, and 45DC was DGGE marker, enriched cultures from cattle dung, horse dung, soil sediment, biogas effluent sludge, and 45 days fermented compost, respectively.

Table 3
EFB biodegradation efficiency and volatile fatty acids analysis for microbial consortia (MC). Values are means ± standard error.

MC	Soluble metabolites ($g \cdot L^{-1}$)						EFB degradation efficiency (%)
	AA	PA	IBA	BA	IVA	VA	
CD	0.187 ± 0.03	0.091 ± 0.00	0.017 ± 0.00	0.098 ± 0.02	0.026 ± 0.00	0.003 ± 0.00	57.5 ± 1.8
HD	0.248 ± 0.08	0.179 ± 0.06	0.034 ± 0.01	0.205 ± 0.08	0.046 ± 0.01	0.006 ± 0.00	48.9 ± 1.9
EF	0.194 ± 0.05	0.094 ± 0.02	0.017 ± 0.00	0.115 ± 0.03	0.025 ± 0.00	0.004 ± 0.00	51.1 ± 9.0
SD	0.257 ± 0.09	0.133 ± 0.04	0.023 ± 0.00	0.164 ± 0.06	0.030 ± 0.01	0.004 ± 0.00	55.1 ± 0.6
45DC	0.553 ± 0.26	0.280 ± 0.12	0.049 ± 0.02	0.342 ± 0.17	0.064 ± 0.02	0.009 ± 0.00	45.2 ± 2.4

Denoting letters: AA: acetic acid; PA: propionic acid; IBA: isobutyric acid; BA: butyric acid; IVA: isovaleric acid; VA: valeric acid.

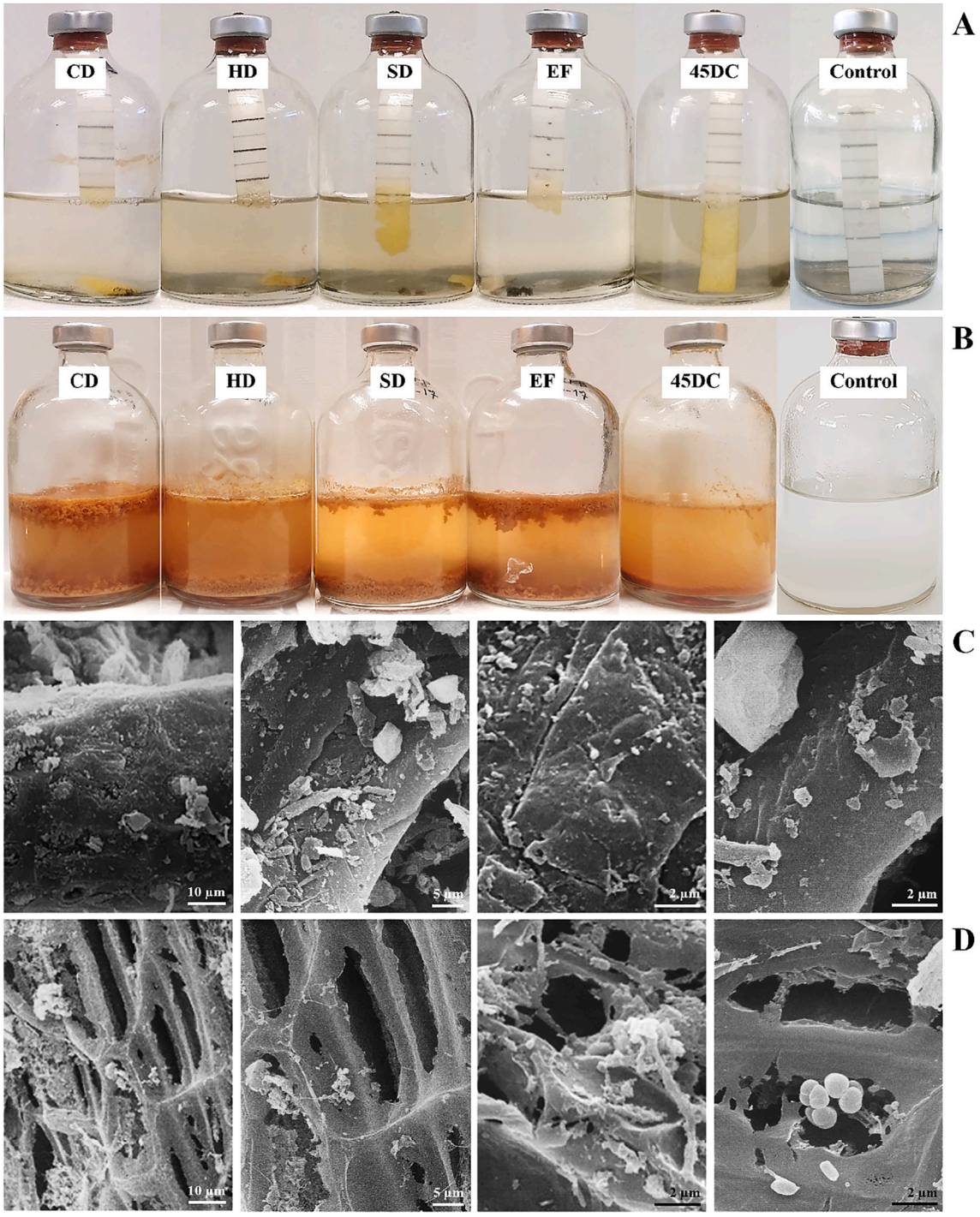

Fig. 2. The filter paper (A) and microcrystalline cellulose (B) degradation of each enriched consortia and scanning electron microscopy (SEM) photograph of non-digested EFB (C) and digested EFB (D) by CD consortium.

structural polysaccharides (Klocke et al., 2007). The CD consortium was considered the best EFB degrader in terms of enzyme activities and EFB degradation, as shown in Fig. 2C–D. Non-digested EFB (Fig. 2C) presents with rough and recalcitrant surface structure. After fermentation with the CD consortium, the bacterial cells were colonized on EFB and degraded EFB resulting big hole (Fig. 2D).

3.3. Augmentation of Bacteroides and Clostridium-rich methanogenic consortium in the AD process

Methane production from anaerobic co-digestion of EFB and POME with control, CD, HD, EF, SD, and 45DC consortium augmentation was 47.2, 79.0, 74.8, 77.2, 74.3, and 71.4 m^3-CH_4·tonne-EFB^{-1} with production rates of 5.9, 11.1, 10.2, 10.7, 10.1, and 10.5 mL-CH_4·d^{-1}, respectively. The hydrolysis constant (k_h) of EF, HD, CD, SD, and 45DC consortium in anaerobic co-digestion of EFB and POME were 0.1059, 0.1183, 0.1151, 0.1116, 0.1175, and 0.1230 d^{-1}, respectively (Table 4). Methane yield was increased by 51.3–67.2% compared with the control (non-augmentation), while the methane production rate was 2-fold higher than control. The CD consortium showed the highest efficiency in enhancing biogas production and was selected for further simulation in the AD reactor. During the first 10 days of incubation, methane production from augmentation treatment was similar for control, indicating that the microorganisms consumed the easily degradable substrate during this period (Fig. 3). After day 10, methane productions of the augmentation treatments were significantly higher than ($p < 0.001$) the control. The changes in the microbial community in AD systems after augmentation is shown in Fig. 4A–B. The microbial community of enriched consortium augmented AD systems were composed of *Bacteroides*, *Clostridium*, *Stenotrophomonas*, *Syntrophomonas*, *Acidobacterium*, *Silanimonas*, *Lactobacillus*, *Weissella*, *Roseomonas*, and *Roseiarcus* sp. These genera have generally been found in high solid AD systems. The saccharolytic *Acidobacterium* sp. was reported to be involved in microbial degradation of lignocellulosic plant biomass and dominated soils rich in organic matter (Rawat et al., 2012). *Syntrophomonas* sp., a bacterium involved in β-oxidization of saturated fatty acids into acetate or acetate and propionate, was found in all the augmented treatments except for the EF-augmented system. *Syntrophomonas* sp. was grown in mutualism with *Desulfovibrio* sp. and methanogens (McInerney et al., 1981). Two genera of *Alphaproteobacteria* that included *Roseiarcus* and *Roseomonas* were found in our augmented AD systems. *Roseiarcus* sp. is known to produce propionate, acetate, and H_2 as primary soluble metabolites (Kulichevskaya et al., 2014). It was found only in HD- and SD-augmented AD systems. *Roseomonas* sp. produced acetic acid by oxidizing sugars or ethanol during fermentation (Komagata et al., 2014) and was found only in control AD systems. By contrast, the *Gammaproteobacteria*, *Silanimonas*, and *Stenotrophomonas* were exiguous in all AD systems. *Bacteroides* and *Clostridium* were the predominant genera in all AD systems. These genera are well-known as cellulase and xylanase producers (Robert et al., 2007; Thomas et al., 2014). The archaeal profile in all augmented AD systems was mainly composed of *Methanosaeta*, *Methanobacterium*, *Methanosarcina*, *Methanoculleus*, and *Methanosphaera* sp. *Methanosaeta* is obligated aceticlastic methanogen, which metabolizes acetate as its sole source of energy (Welte and Deppenmeier, 2014). While most *Methanosphaera* reduces carbon dioxide and hydrogen to methane, *Methanobacterium* lives by reducing carbon dioxide, hydrogen, and formate to methane. *Methanoculleus* is a common methanogen in the AD reactor that uses ethanol and secondary alcohol as an electron donor to produced methane (Angelidaki et al., 2011). *Methanosarcina* was not found in the control AD system compared to all augmented AD systems. *Methanosarcina* has both aceticlastic and hydrogenotrophic functions involved in methanogenesis (Welte and Deppenmeier, 2014).

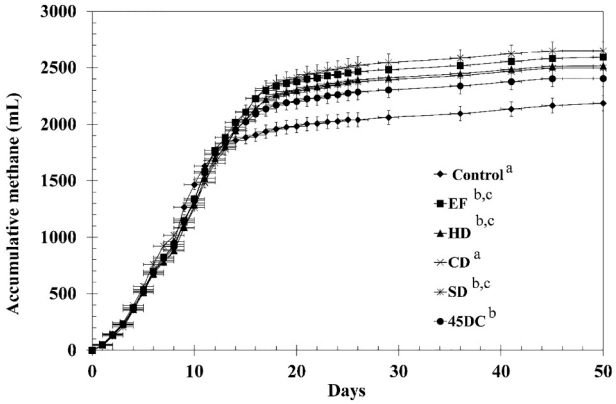

Fig. 3. The enhancement of biogas production from co-digestion of EFB with POME by bioaugmentation with 5 enriched consortia.

3.4. Augmentation of CD consortium in AD process simulation

Augmentation of CD consortium in the AD process simulation as prehydrolysis of EFB (TR1), prehydrolysis of EFB with easy degradation POME (TR2), directly augmented into AD digester tank (TR3), and mixed CD consortium with feedstock in mixing tank before feeding to AD digester tank (TR4) gave methane yields of 342.2, 327.1, 355.5, and 376.7 mL-CH_4·g-VS^{-1}, respectively (Table 5). The methane yield of the control reactor was 203 mL-CH_4·g-VS^{-1}. The TR3 and TR4 showed significant improvement in methane yield of 75.1–85.5% (Fig. 5). Applying the CD consortium for TR1 and TR2 improved methane yields of 61.1–68.5%. The CD consortium improved EFB degradation efficiency from 85.7% to 94.4%. The methane production rates of control, TR1, TR2, TR3, and TR4 reactors were 9.0, 18.3, 19.6, 22.7, and 24.0 mL-CH_4·d^{-1}, respectively. The methane productions of the control, TR1, TR2, TR3, and TR4 reactors were 40.9, 68.7, 65.8, 71.4, and 75.7 m^3-CH_4·$tonne^{-1}$ EFB, respectively. The biogas reactor with CD consortium augmentation in the feedstock mixing tank (TR4) showed a significant increase in biogas production. The methane production rate of the prehydrolysis reactor (TR1 and TR2) and augmentation reactor (TR3 and TR4) was enhanced by 8.3–15.7% and 34.0–41.9%, respectively. The hydrolysis constant was improved in augmentation treatment (TR3 and TR4) with a k_h value of 0.1636–0.1725 d^{-1}. The direct augmentation into the AD reactor (TR3) has methane production and methane production rate lower than augmentation in the mixing tank (TR4). The immediate consumption of easy degradation substrate by indigenous microorganisms resulted in low substrate available for cellulolytic

Table 4
The efficiency of five microbial consortia (MC) by bioaugmentation during co-digestion of EFB with POME for methane production.

Experiments	Methane yield (mL-CH_4·g^{-1} VS)	Methane production rate (mL-CH_4·d^{-1})	Methane production (m^3-CH_4·tonne EFB^{-1})	Degradation efficiency (%)	Methane yield improvement (%)	Hydrolysis constant; k_h (d^{-1})
Without MC	235.5 ± 7.1	5.9 ± 0.2	47.2 ± 1.4	56.3 ± 1.7	0.0 ± 0.0	0.1059 ± 0.003
CD	393.5 ± 11.8	11.1 ± 0.3	79.0 ± 2.4	91.4 ± 2.7	67.2 ± 2.0	0.1183 ± 0.004
HD	372.8 ± 11.2	10.2 ± 0.3	74.8 ± 2.2	89.4 ± 2.7	58.5 ± 1.8	0.1151 ± 0.003
EF	384.4 ± 11.5	10.7 ± 0.3	77.2 ± 2.3	92.1 ± 2.8	63.4 ± 1.9	0.1116 ± 0.003
SD	370.3 ± 11.1	10.1 ± 0.3	74.3 ± 2.2	88.7 ± 2.6	57.3 ± 1.7	0.1175 ± 0.004
45DC	355.6 ± 10.7	10.5 ± 0.3	71.4 ± 2.1	85.3 ± 2.6	51.3 ± 1.5	0.1230 ± 0.004

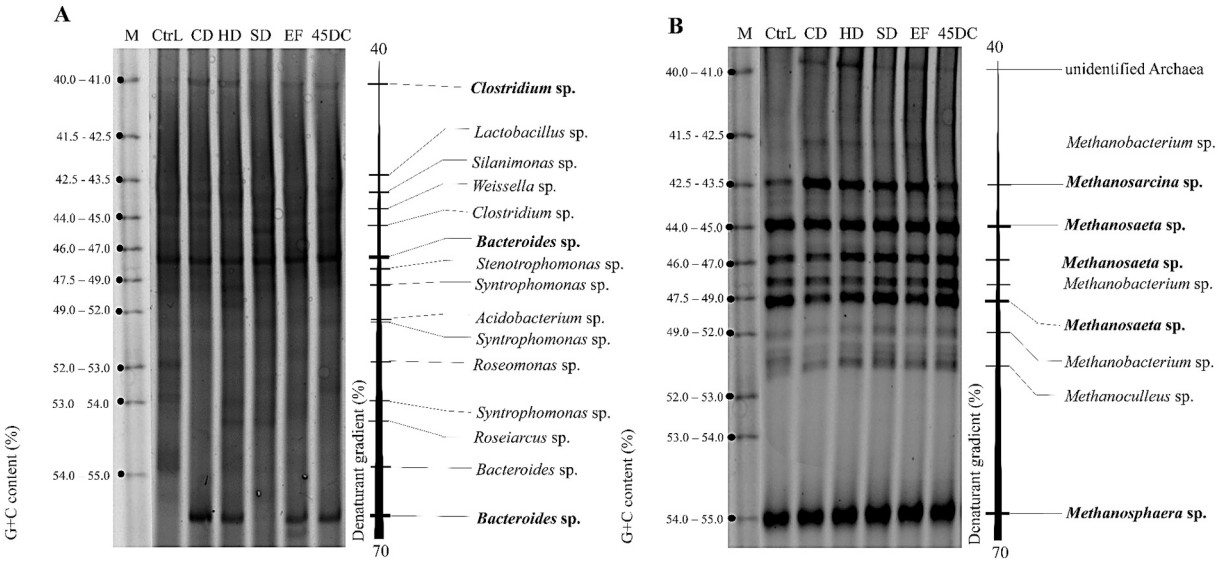

Fig. 4. Bacterial community profiles (A) and archaeal community profiles (B) of augmented biogas system with CD consortium (CD), HD consortium (HD), SD consortium (SD), EF consortium (EF), 45DC consortium (45DC), control (CtrL), and marker (M).

Table 5
The performance of EFB feeds biogas reactors with various applied strategies for *Bacteroides* and *Clostridium*-rich methanogenic sludge.

Experiments	Methane yield (mL-CH_4·g^{-1} VS)	Methane production rate (mL-CH_4·d^{-1})	Methane production (m^3-CH_4·tonne^{-1} EFB)	Degradation efficiency (%)	Methane yield improvement (%)	Methane production rate improvement (%)	Hydrolysis constant; k_h (d^{-1})
Control	203.0 ± 6.1	9.0 ± 0.3	40.9 ± 1.2	50.8 ± 1.5	0 ± 0.0	0 ± 0.0	0.1439 ± 0.004
TR1	342.2 ± 10.2	18.3 ± 0.5	68.7 ± 2.1	85.7 ± 2.6	68.5 ± 2.1	8.3 ± 0.2	0.1444 ± 0.004
TR2	327.1 ± 11.2	19.6 ± 0.6	65.8 ± 1.8	82.0 ± 2.5	61.1 ± 1.8	15.7 ± 0.5	0.1475 ± 0.004
TR3	355.5 ± 10.7	22.7 ± 0.7	71.4 ± 2.1	89.1 ± 2.7	75.1 ± 2.3	34.0 ± 1.0	0.1636 ± 0.005
TR4	376.7 ± 11.3	24.0 ± 0.7	75.7 ± 2.3	94.4 ± 2.8	85.5 ± 2.6	41.9 ± 1.3	0.1725 ± 0.005

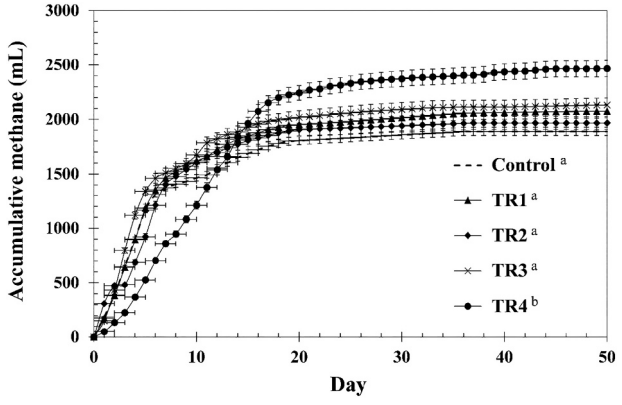

Fig. 5. Biogas production from the AD simulation test of augmented with CD consortium (solid fill) treatment 1 (TR1), treatment 2 (TR2), treatment 3 (TR3), and treatment 4 (TR4) compared to unaugmented (airy fill).

bacteria. The augmentation in the mixing tank (TR4) could increase both indigenous and exogenous microorganisms that break down the easy-to-hard substrate. TR4 treatment also an increased number of hydrolytic bacteria and allows more lignocellulosic substrates to be digested. The biogas production from bioaugmentation was higher than control. The energy balance and economic feasibility of *Bacteroides* and *Clostridium*-rich methanogenic consortium augmentation for enhancing biogas production from anaerobic c-digestion of EFB and POME was carried out in this study (Table 6). The possibility of the extra cost,

Table 6
Energy and economic evaluation for the anaerobic co-digestion of EFB and POME with bioaugmentation.

Energy parameters	Bioaugmentation reactor (TR4)	Control reactor
Microbial cultivation (kWh·kg^{-1} TS)	0.77	0
- Medium for microbial cultivation (kWh·kg^{-1} TS)	0.51	0
- Agitation (kWh·kg^{-1} TS)	0.26	0
Bioaugmentation process (kWh·kg^{-1} TS)	0.822	0.76
- Pump (kWh·kg^{-1} TS)	0.76	0.76
- Agitation (kWh·kg^{-1} TS)	0.062	0
Energy input (kWh·kg^{-1} TS)	1.59	0.76
Energy from biogas production (kWh·kg^{-1} TS)	5.76	3.10
Net energy (kWh·kg^{-1} TS)	4.17	2.34
Additional energy gain (kWh·kg^{-1} TS)	2.66	–

electrical energy, the profit accrued from the sale of the additional electricity being sufficient to cover the cost of microbial cultivation, and the cost of bioaugmentation strategy. The energy of 1.59 kWh·kg^{-1} TS was added to the bioaugmentation reactor. The additional energy of 2.66 kWh·kg^{-1} TS was achieved from biogas production of bioaugmentation reactor. The net energy balance (4.17 kWh·kg^{-1} TS) of the bioaugmentation reactor was two times higher than the control reactor.

The microbial community from next-generation sequencing analysis of the CD consortium (*Bacteroides* and *Clostridium*-rich methanogenic consortium) revealed that the predominant bacterial orders belonged to *Bacteroidales*, *Clostridiales*, and *Synergistales*, in agreement with the

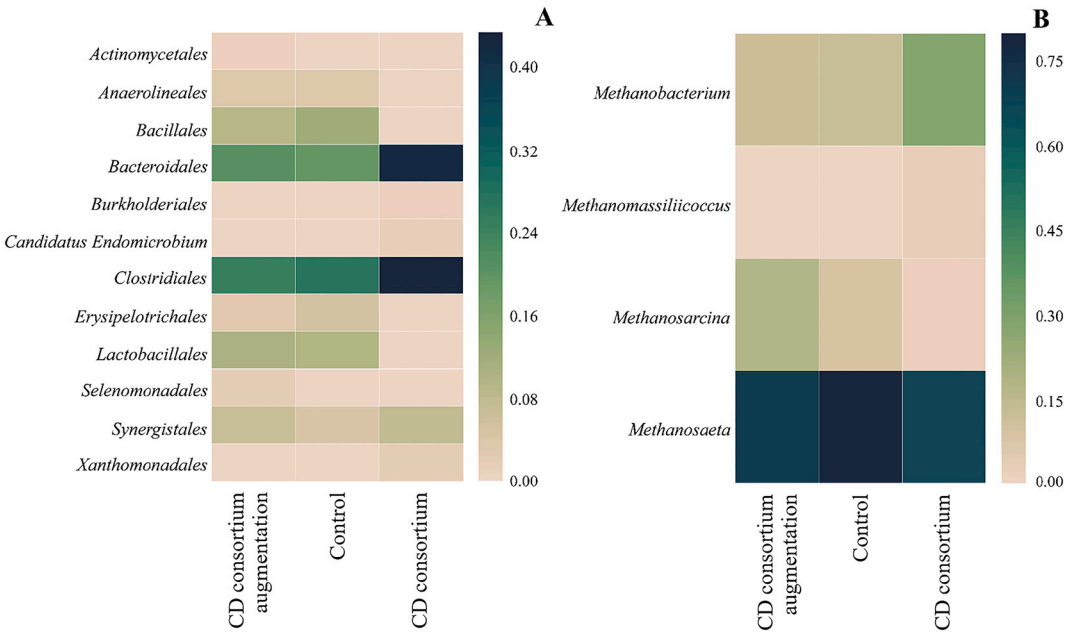

Fig. 6. Bacterial community changes in the order-level taxonomic composition (A) and archaeal community changes in the genus-level taxonomic composition (B) of CD consortium-augmented biogas system compared to the unaugmented biogas system.

DGGE results. At the same time, the archaeal community was dominated by *Methanosaeta* and *Methanobacterium* (Fig. 6). The AD sludge without augmentation was dominated by *Clostridiales*, *Bacillales*, *Bacteroidales*, and *Lactobacillales*, while *Methanosaeta*, *Methanosarcina*, and *Methanobacterium* dominated the archaea. The numbers of *Clostridiales*, *Bacteroidales*, and *Synergistales* increased in the CD consortium augmented AD reactor. The bacterial community of the TR4 reactor was composed of *Clostridiales*, *Bacteroidales*, *Synergistales*, *Lactobacillales*, and *Bacillales*, while *Methanosaeta*, *Methanosarcina*, and *Methanobacterium* were dominated in the archaea community. Species of the genus *Methanosarcina* have a broad spectrum of substrates compared to other methanogens, with the ability to use acetate, methanol, and other methylated C_1 compounds for methane formation. Some genus *Methanosarcina* can also utilize $H_2 + CO_2$ for methane formation (Welte and Deppenmeier, 2014). *Methanobacterium* sp. is a hydrogenotrophic methanogen dominated in CD consortium and decreased in the augmented AD reactor. The most abundant methanogens *Methanobacteriales* and *Methanomicrobiales* were found in the cellulolytic consortium augmented batch fermentation (Ozbayram et al., 2017). Tsapekos et al. (2017) reported that bioaugmentation did not markedly alter indigenous microbial communities. This result differed from our study, especially for the methanogen population. Our results provided evidence that the system was rich in acetic acid-producing bacteria, leading to a high abundance of aceticlastic methanogens. This result concurred with the fact that aceticlastic methanogens favored high organic acid concentration environments (Zhang et al., 2011). The CD consortium showed diversified methanogen populations related to the bacterial community. Synergistic microorganisms from the CD consortium promoted hydrolyze lignocellulosic biomass, releasing VFAs and methanization that ultimately increased biogas productivity. *Bacteroides* and *Clostridium*-rich methanogenic consortium augmentation were tested in the batch reactor to confirm the performance, existence capacity, and effect on indigenous microorganisms in the AD system before applying it into a long-term continuously-fed reactor. However, it still needs to prove in the long-term continuously fed reactor for the loss of bio-augmentation species by competition with the indigenous microorganism and dilution rate.

4. Conclusions

The *Bacteroides* and *Clostridium*-rich methanogenic consortium were successfully enriched by xylan containing medium with high xylanase and cellulase activities and enhanced biogas production of high-solid anaerobic digestion (AD) system. The augmentation of *Bacteroides* and *Clostridium*-rich methanogenic consortium in the feedstock mixing tank before a feed to the AD reactor was significantly increased methane production by 67.2% compared to the non-augmentation. The net energy balance (4.17 $kWh \cdot kg^{-1}$ TS) of the bioaugmentation reactor was two times higher than the non-augmentation reactor. Bioaugmentation of *Bacteroides* and *Clostridium*-rich methanogenic consortium promoted hydrolytic activity and balancing the methanogenic community in the high-solid AD system.

CRediT authorship contribution statement

Wisarut Tukanghan: Conceptualization, Methodology, Writing-original draft.

Sebastian Hupfauf: Leader for microbial bioinformatics data analysis.

María Gómez-Brandón: Leader for qPCR analysis.

Heribert Insam: Visualization, Writing-review & Editing.

Willi Salvenmoser: Leader for Electron microscopy analysis.

Sompong O-Thong: Visualization, Writing-review & Editing.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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