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Dry Anaerobic Digestion of Cow Dung for Methane Production: Effect of Mixing

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Abstract: The performance characteristics of a dry batch reactor with a blender treating cow dung has been evaluated for 35 days in a single-stage batch reactor of 3 L effective volume at 35±1°C to investigate the effect of continuous-mixing on biogas production and organic materials removal. The results showed that the performance of unmixed and mixed digesters was quite different and the dry digester with mixing system produced methane of 0.358 LCH_4/gVS_r which was 7.50% higher than that for unmixed digester. Moreover, the organic material removal efficiency was increased by 9.73% in term of VS. The wide diversity of prominent bacteria and methanogenic archaea affiliated with all steps along the anaerobic degradation pathway made the process stable. But the dry digester with mixing system during start up was not beneficial, as it resulted in relatively higher volatile fatty acids, higher volatile fatty acid to alkalinity ratio, lower pH and consequently prolonged start up time.

Key words: Dry anaerobic digester, Biogas, organic materials removal

INTRODUCTION

Dry anaerobic digestion is an alternative to conventional manure management, alleviating health and environmental concerns and converting organic wastes (TS>10%) by microbial consortia in oxygen-free environment into biogas (Jha *et al*., 2011). Besides reactor type, retention time, loading rate, quality of feedstocks, environmental conditions within the digester and other related parameters, the performance of an anaerobic digester depends upon the degree of contact between the substrate and a viable bacterial population (Karim *et al*., 2005; Kalia and Singh, 2001).

Adequate mixing can enhance biogas production and biodegradability (Kalia and Singh, 2001) due to the distribution of substrates, enzymes and micro-organisms throughout the digester. Mixing also promotes heat transfer and particle size reduction, discharges gas bubbles trapped in the medium and avoids the sedimentation of denser particulate matter (Kaparaju *et al*., 2008; Ward *et al*., 2008). Although many researches (Karim *et al*., 2005; Kaparaju *et al*., 2008; Kalia and Singh, 2001) have reported about the importance of mixing in achieving efficient substrate conversion in wet anaerobic digesters, there is not a clear picture about the consequence of mixing on dry anaerobic digestion of manure. In the present study, the effect of continuous mixing on dry anaerobic digestion of cow dung for biogas production and organic materials removal has been investigated.

MATERIALS AND METHODS

Experimental set up and procedure: The experiment was conducted into a single-stage batch reactor with mixing system at 35±1°C. The capacity of the reactor was 3.6 L with 3 L effective volume (Fig. 1). The produced biogas was escaped through a pipe into a water lock (3.0 pH) and then into a wet gas meter and finally released into the atmosphere. The samples for detecting various parameters were taken out from the side-ports. The samples were stored at -4°C in a freezer before analysis. In the fermentation process, the substrate was fed into the airtight digester under specified environmental conditions for 35 days without dilution. The digester was purged with nitrogen for 15-20 min to create complete anaerobic environment.

Characteristics of feedstocks: The cow dung was obtained from a livestock farm of Harbin, China and made free from foreign materials including stone, wood, metal, straw, feather and other inorganic materials, manually. The cow dung was then inoculated with the 20% mesophilic digestate obtained from the previous investigation of dry

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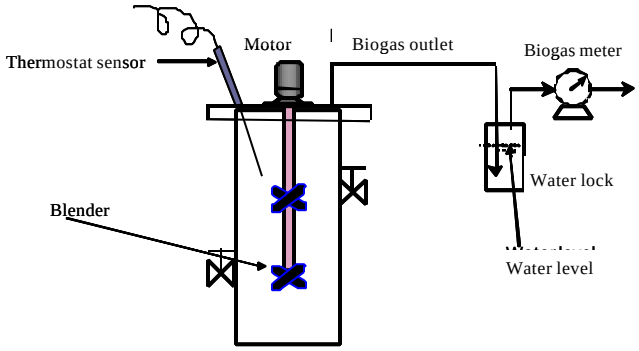

Fig. 1: Schematic diagram of the batch reactor with mixing system

anaerobic digestion of cow dung. The cow manure contained 16.28% total solids (TS),130.21 g Volatile Solids (VS), 145.75 g Chemical Oxygen Demand (COD), 59.83 g soluble COD, 2.87 g L^{-1} nitrogen, 1.41 g L^{-1} phosphorus, 1.47 g L^{-1} ammonia nitrogen and 0.85 g L^{-1} free ammonia per kg of the wet-manure. The high proportion of VS to TS (84.3%) depicts that a large fraction of the manure was biodegradable and could serve as an important feedstock for biogas production. The C: N of the manure was found adequate (25:1) because it is often suggested that the C: N ratio in the substrate should be in between 20:1 to 30:1. The pH of the manure was also found favourable for the anaerobic digestion. In this study, 3 kg cow manure and 0.60 kg inoculant were mixed and incubated into the air-tight dry digester with mixing system.

Analytical methods: The physico-chemical parameters analyzed were temperature, pH, TS, VS, COD, soluble COD, Volatile Fatty Acids (VFAs), nitrogen, phosphorus, ammonia nitrogen and free ammonia. All the analytical determinations were performed according to the standard methods (APHA, 1995). All the tests were conducted in triplicate and mean values were reported. The pH was measured with a digital pH meter (Model 526, Germany). Free ammonia was calculated using the formula described in the previous study (Ostergaard, 1985). The yielded biogas was measured daily using wet gas meter (LML-1, Changchun Co. Ltd). The constituents (CH_4, CO_2 and H_2) of the biogas were determined using Gas Chromatography (SC-7, Shandong Lunan Instrument Factory). The samples taken from the batch culture was centrifuged at 6,000 rpm for 15 min and then acidified with hydrochloric acid and filtered through a 0.2 μm membrane for the analysis of VFAs and ethanol. The concentrations of the VFAs and ethanol were determined using a second gas chromatograph (Model GC122, Shanghai Analysis Instrument Factory).

Microbial community analysis: Genomic DNA of the sludge samples was extracted using a DNA extraction Kit (MO Bio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. Extracted DNA was dissolved in 60 μL 1×TE buffer solution. The V3 and V4 regions of 16S rRNA were amplified by PCR using universal bacterial primers (341F, 5'-CCTACGGGAGGCAGCAG-3' with a GC clamp and 907R, 5'-CCGTCAATTCMTTTGAGTTT-3') and universal archaeal primers (344F, 5'-ACGGGGYGCAGCAGGCGCGA-3' with a GC clamp and 915R, 5'-GTGCTCCCCCGCCAATTCCT-3'). The PCR amplification was conducted in a 50 μL system containing 5 μL 10 × Ex Taq buffer, 4 μL dNTP mixture (2.50 mM), 1 μL forward primer (20 μM), 1 μL reverse primer (20 μM), 2.5 ng DNA template and 0.15 U Ex Taq DNA polymerase (Takara, Dalian, China), using a thermal cycler (model 9700; ABI, Foster, CA, USA), started with an initial denaturation of DNA for 10 min at 94°C, followed by 30 cycles for 1 min at 94°C, 30 sec at 55°C (decreasing by 0.10°C per cycle to 52°C) and 1 min at 72°C; final extension was 10 min at 72°C. The PCR products were separated using the Dcode™ universal mutation detection system (Biorad Laboratories, Hercules, CA, USA). Polyacrylamide gels with 40-60% vertical denaturing gradient were prepared. The 10 μL PCR products were loaded and electrophoresed at 120 V and 60°C for 10 h. Gels were stained silver as described in the previous research (Bassam *et al*., 1991). All DGGE bands were excised and dissolved in 30 μL 1×TE at 40°C for 3 h and then centrifuged at 12000 rpm for 3 min. The 3 μL supernatant was used as the template and conducted PCR amplification under the conditions as above described using the same primers. The PCR products were pured by Gel Extraction Mini Kit (Watson biotechnologies. Inc, China) and ligated into pMD18-T vector (Takara, Dalian, China) and then cloned into *E. coli* DH5α. Some white clones from each sample

were randomly selected for PCR detection and positive clones were selected for sequencing by ABI3730 and partial 16s rRNA gene sequences were analyzed using the BLAST program in GenBank at National Center for Biotechnology Information <http://www.ncbi.nlm.nih.gov/BLAST>.

RESULTS AND DISCUSSION

Process characteristics: Figure 2 presents evolution of pH, NH_3-N, free ammonia and VFAs. The pH of cow dung was initially around 7.64. Though pH and temperature are homogeneously mixed inside the digester with mixing system, the pH variation pattern was observed identical to the unmixed system. That means the pH was decreased swiftly during start up phase of the experiment due to the increase in VFAs production by acidogenic bacteria as well as carbonic acid associated with the high concentrations of carbon dioxide gas. The easily digestible fraction of organic matter was hydrolyzed and converted to fatty acids rapidly. The decrease in pH value was observed more in the reactor with mixing system than that in the unmixed reactor. The main reason for the lower pH during the mixing was attributed to relatively high production of VFAs as well as release of the H^+ ions during ammonia stripping. This result is consistent with the previous research (Kaparaju *et al*., 2008) which stated that vigorous mixing disrupts the structure of microbial flocks and affect digestion efficiency. The pH was began to rise gradually as the VFAs were consumed by methanogens and transferred to the methane. It was also observed that there was stable pH after 4th week. The substrate was able to buffer itself and prevent the acidification occurrence during digestion due to proper alkalinity of cow dung, which is a pre-requisite for proper biogas production. The phosphorus, nitrogen and ammonia nitrogen were noted 1.41 to 1.13, 2.87 to 1.79 and 1.52 to 1.43 g L^{-1}, which are sufficient to satisfy the cell growth requirements for biogas production. Variation in ammonium nitrogen levels was relatively lower compared to unmixed system because of stripping of ammonia in continuous mixing of the substrate inside the digester. The ammonia concentrations were noted below the inhibitory levels as the critical ammonia concentration is 2.8 g L^{-1} (Poggi-Varaldo *et al*., 1997).

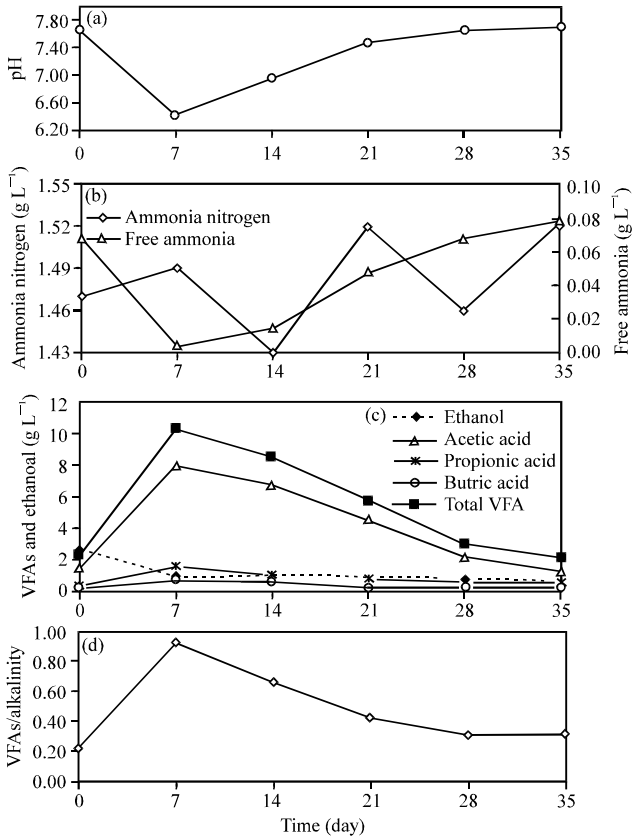

Fig. 2(a-d): Evolution of (a): pH, (b): Ammonia nitrogen and free ammonia, (c): VFAs and (d): VFAs/alkalinity in the reactor with mixing system

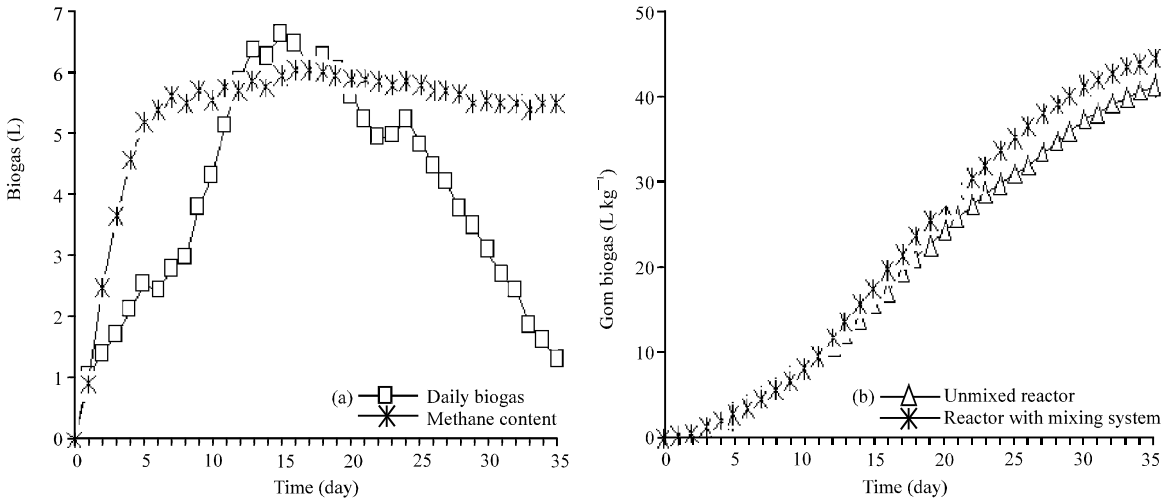

Fig. 3(a-b): (a): Daily biogas production and its methane content in the reactor with mixing system and (b): Comparison of cumulative biogas yields in the reactors with or without mixing system

Free ammonia is the active component causing ammonia inhibition (Hansen *et al*., 1998). The calculated free ammonia ranged from 0.01 to 0.08 g L^{-1} in the reactor, indicating no possibility of inhibition in the process due to existence of free ammonia in the reactor with blending system. The value obtained was not supposed to be high enough to create inhibition as though ammonia can inhibit fermentation process; the total ammonia concentration that can be tolerated was relatively high. Volatile fatty acids are usually produced due to the degradation of the complex organic polymers during hydrolysis and acidogenic stages. The conversion of intermediate products-VFAs-has been treated as an indicator of the digestion efficiency but the high concentration of VFAs results in decrease of pH, inhibit acidification, destroy methanogenic bacteria activity and leading to failure of digester ultimately. In this study, the reactor showed high volatile fatty acids concentrations in the start up phase (Fig. 2c) due to higher acidogenesis and lower methanogenic activities. The principal volatile fatty acids formed were acetic, butyric and propionic acids. Acetic acid was the dominant volatile fatty acid. The share of propionic and butyric acids was observed low because of the sufficient propionate- and butyric-degrading syntrophs which could rapidly convert propionic acid and butyric acid to acetic acid (Montero *et al*., 2008). The VFAs were increased rapidly after starting the test and reached a maximum of 10.33 g L^{-1} after 7 days. During this period, the acetic acid production rate was apparently higher than the acetic acid consumption rate. The degradation of propionate and butyrate by syntrophic acetogenic bacteria produced acetic acid that was subsequently degraded into methane and CO_2 by acetoclastic methanogens (Montero *et al*., 2008). During methanogenic stage, acetic acid was started to convert into biogas such as methane and carbon dioxide. Thus, as methanogenesis and methane gas yield have increased, the VFAs concentrations were decreased. No high VFAs accumulation was detected due to perhaps acetatoclastic methanogens could consume acetate quickly in the digester to yield methane and carbon dioxide. At the end of the process, VFAs concentration was decreased to 2.18 g L^{-1}. The ratio of VFAs to alkalinity was in favorable range during the digestion period but it was more than 0.80 on the 7th day. It means the reactor was slightly inhibited during the first week (Sanders *et al*., 1996). Afterwards, the process seemed stable as no accumulation of VFAs and fall in pH were observed and consequently no significant inhibition during the digestion period.

Enhancement of biogas production and biodegradability: Figure 3 shows the daily biogas production and its methane content in the reactor with mixing system. The biogas generation was started after seeding, kept increasing until reaching the peak and then began to decline. The biogas production and methane yield patterns were found similar to the previous unmixed mode (Jha *et al*., 2010; Li *et al*., 2011). It means the biogas and methane generation showed an increase day after day until reached the maximum value and then decreased slowly day after day but biogas production was detected low in the reactor with mixing system during first week of the fermentation process. It happened due to high VFAs production, decrease in pH value and lack of methanogens. This result indicated that the reactor with

Table 1: Comparison of characteristics of mixing system with unmixed reactor

Reactors	Cumu. biogas (L kg^{-1})	Cumu.methane (L kg^{-1})	Average methane content (%)	VS removal (%)	COD removal (%)	Methane yield	
						L CH_4/VS_r	L CH_4/COD_r
Reactor with mixing system	47.56	26.72	56.19	41.90	45.26	0.358	0.298
Unmixed reactor	44.10	25.21	57.04	38.19	41.28	0.333	0.271

r: Removal

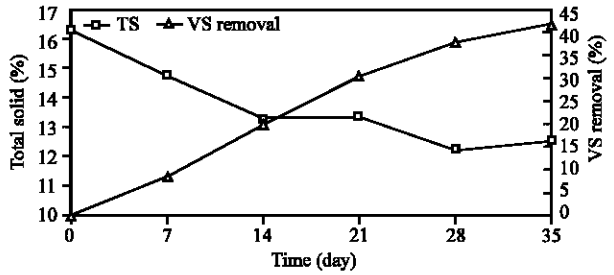

Fig. 4: Total solid and removal of VS in the reactor with mixing system during the fermentation period

the mixing system for treating cow dung could not be favourable during the start up period. The cumulative biogas generation was measured 47.56 with 26.72 L kg^{-1} methane content during 35 days of the digestion period. Comparing with the previous unmixed bioreactor (Table 1), additional 7.50% biogas was noted in the reactor with mixing system. The percentage of extra biogas was not so high because the unmixed reactor was also actually mixed 2-3 minutes daily. No significant change was observed in the quality of biogas.

The initial methane contents in the yielded biogas has increased and exceeded to the peak value and then decreased slowly to some extent. The hydrogen content in the biogas was observed negligible as like the unmixed bioreactor. Figure 4 also presents VS removal efficiency in the reactor with mixing system during the digestion period. The VS was degraded about 41.90% in the reactor with mixing system. It was degraded about 38.19% in the previous study of dry fermentation process of cow dung in the reactor without mixing system for the same digestion period (Li *et al*., 2011). Thus, the mixing strategy could boost VS removal efficiency by 9.73%. The VS removal has close relation with the methane yield. Therefore, the percentage of VS removal was increased as cumulative methane production was increased.

Microbial community analysis: The sludge was sampled for PCR-DGGE analysis on the seventh day and at the end of fermentation process (digestate) to understand the potential linkage between the bacterial and archaeal community structure and digestion process performance. The thirteen prominent bands were obtained from DGGE for bacteria (Fig. 5a) and then sequenced. The phylogenetic analysis of the representative bacterial clones revealed that micro-organisms in the phyla Firmicutes, Bacteroides, Proteobacteria and Ruminobacillus were observed (Table 2). Among them, the phylum Firmicutes was dominant group and within phylum Firmicutes, class *Clostridia* was the most dominant of the bacterial community. Micro-organisms within the class *Clostridia* and *Bacteroidetes* have been frequently reported to be important throughout various anaerobic habitats and have the ability to degrade a wide variety of complex organic molecules, including proteins and carbohydrates. *Clostridum* and *Bacteroides* species isolated from rumen, digesters and natural habitats hydrolyze cellulose, hemi-cellulose and protein to produce VFAs, alcohol, CO_2 and H_2. Most of the members of the genus *Clostridium* are strictly anaerobic, producing ammonia, H_2S and H_2 and ferment carbohydrates into acetate, butyrate, ethanol, CO_2 and H_2. Therefore, the higher VS reduction, obtained might be due to the high abundance of these bacteria. In this study, the identified micro-organisms within these classes are in agreement with other community analyses in anaerobic digesters and demonstrate the importance of these phylogenetic groups, for the degradation of complex organic matter in the fermentation process systems. There were eleven prominent bands obtained from DGGE in archaea domain (Fig. 5b) and PCR-DGGE patterns of different DNA fingerprint bands represented the different microbial species of the 16S rRNA. DGGE profile clearly reflected the shift in microbial communities and the number of bands observed at the end of experiment for archaea was more than those for the initial stage (on the 7th day). Euryarchaeota was the most abundance archaeal 16S rRNA gene sequences and classified into Methanomicrobiales and Methanosarcinales. Hydrogenotrophic methanogens, such as *Methanoculleus*, *Methanogenium* and

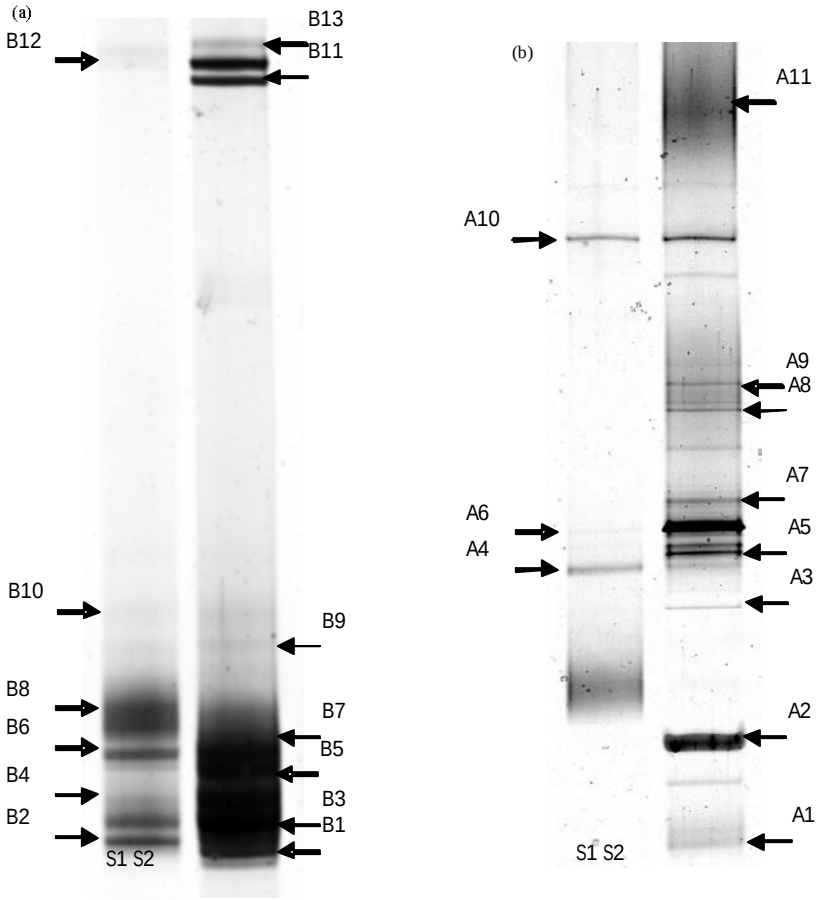

Fig. 5: DGGE fingerprints of the samples (a) Bacteria and (b) Archaea in the reactor with mixing system, (S1): Sample of the 7th day, (S2): Digestate

Table 2: Bacterial 16S rRNA gene clone libraries in the dry methane fermenter with mixing system, compared by BLAST with NCBI

Taxonomy	Band	Accession No.	Closest sequence	Identity (%)
Firmicutes	B2	AB298753	*Clostridiaceae bacterium* SK061 gene	93
	B4	DQ445863	*Eubacterium tenue strain* ATCC 25553 clone	99
	B7	NR029239	*Clostridium chartatabidum* strain 163	99
	B11	EU728789	*Ruminococcus sp.* DJF_VR52	99
	B12	NR042436	*Lactobacillus coleohominis* DSM 14060 strain: DSM 14060	99
	B13	JN944720	*Streptococcus gallolyticus* strain ULAG59	99
Bacteroidetes	B1	AY158021	*Prevotella* sp. RS2	93
	B3	HQ020488	*Parabacteroides* sp. Lind7H	99
	B5	GU112991	*Rikenellaceae bacterium* 4-1-11	97
	B6	CP000867	*Methanococcus maripaludis* C6	94
Proteobacteria	B8	EF471233	*Alcaligenes sp.* BBTR16	99
Ruminobacillus	B9	DQ178248	*Ruminobacillus xylanolyticum*	96
	B10	DQ168648	*Bacterium* JN18.A7.F	99

Methanobrevibacter, dominated the archaeal communities along with some aceticlastic methanogens. *Methanoculleus bourgensis*, *Methanosarcina barkeri*, *Methanospirillum hungatei* and *Methanomicrobiales* archaeon were the most abundant methanogenic species in the fermenter (Table 3). Each of them showed some specific characteristics in methanogenic metabolism. *Methanoculleus bourgensis* was reported to use H_2-CO_2 or formate as a substrate for growth and methanogenesis and is a hydrogenotrophic methanogen. *Methanospirillum hungatei* produces methane only from H_2-CO_2 or formate, but not from acetate or ethanol and methanol, being a strictly hydrogenotrophic methanogen. *Methanosarcina barkeri* could be used in different substrates to produce methane, including H_2-CO_2, methanol, mono-, di- and trimethylamines, acetate and CO

Table 3: Archaeal 16S rRNA gene clone libraries in the dry methane fermenter with mixing system, compared by BLAST with NCBI

Taxonomy	Band	Accession No.	Closest sequence	Identity (%)
Euryarchaeota (Methanomicrobiales)	A3	AB517987	*Methanospirillum hungatei* gene	99
	A5	EU863828	*Methanogenic archaeon* LGM-AFM04	99
	A6	AB065298	Methanoculleus bourgensis gene	97
	A10	DQ280483	*Methanomicrobiales archaeon*	97
Euryarchaeota (Methanosarcinales)	A1	AJ002476	Methanosarcina barkeri str. CM1 16S rRNA *gene*	97
	A2	DQ987528	*Methanosarcina mazeii* strain LM5	92
	A9	X51423	Methanothrix soehngenii DNA	99
Euryarchaeota (Methanobacteriales)	A4	HM125682	Uncultured *Methanobrevibacter* sp. clone RJ5	95
	A11	DQ135988	*Methanobrevibacter* sp. 1Y	99
Crenarchaeote	A8	AP011757	Uncultured *prokaryote*	86
Euryarchaeota	A4	EF552190	Uncultured *euryarchaeote* clone	98

and is a hydrogenotrophic or aceticlastic methanogen. The prominent bacteria and methanogenic archaea that can be affiliated with all steps along the anaerobic degradation pathway of organic matter to methane have been detected. The high diversity and dynamic activity of methanogens is favorable for maintaining the efficiency of the fermentation process.

CONCLUSION

Mixing appeared to be necessary for effective operation of biogas plants and consistency of feedstock fermentation inside the reactor because it created close connection between micro-organisms and substrate, even distribution of pH and temperature. High diversity of bacteria and archaea supported favorable anaerobic condition within the reactor. Though the performance of the dry methane fermenter with mixing system has poor in the first week due to higher production of VFAs and consequently lower pH, continuous mixing strategy had provided 7.50% additional methane yield and 9.73% higher volatile solid removal efficiency during the fermentation period of 35 days compared to the unmixed dry methane fermenter. In order to prevent higher accumulation of VFAs during start-up phase, intermittent-mixing may be useful.

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