Microorganism population in two-phase anaerobic fermentation of separated liquid of dairy manure

Qiang Li, Zhengjun Guan*, Guoxiang Zheng

(*College of Engineering, Northeast Agricultural University, Harbin 150030, China*)

Abstract: In order to reduce incomplete fermentation caused by high substrate viscosity and low mass transfer efficiency during fermentation process, batch and two-phase anaerobic fermentation experiments were conducted in this study. Dairy manure was separated by using solid-liquid separator firstly. Separated liquid (SL) and diluted dairy manure (DDM) as raw materials were evaluated in terms of gas production performance for both batch and two-phase anaerobic fermentation. The microorganism population was characterized by scanning electron microscope (SEM) and Denaturing Gradient Gel Electrophoresis (DGGE). The results showed that Volatile Solid (VS) methane yield of SL was 124.51 L/kg VS, which was 2.09 times higher than that of DDM (59.50 L/kg VS) in batch anaerobic fermentation. The *Bacteroides* and *Veillonella* with higher activity were the majority microorganism population in acidogenic phase, whereas the *Firmicutes* and *Corynebacterium* with methanogenic properties became the predominant microorganism population in methanogenic phase. This study achieved the phase separation and improved the gas production performance.

Keywords: separated liquid, diluted dairy manure, two-phase anaerobic fermentation, microorganism, scanning electron microscope (SEM), Denaturing Gradient Gel Electrophoresis (DGGE)

DOI: 10.25165/j.ijabe.20181101.3206

Citation: Li Q, Guan Z J, Zheng G X. Microorganism population in two-phase anaerobic fermentation of separated liquid of dairy manure. Int J Agric & Biol Eng, 2018; 11(1): 206–211.

1 Introduction

With the rapid development of Chinese livestock industry, the environmental pollution caused by livestock and poultry gains more attention. Waste heap and discharge have a direct or indirect negative impact on water, air and soil for human health and ecosystems[1-3], as well as the wasting of organic matter. Anaerobic fermentation is an effective approach to transfer livestock and poultry waste to energy and value-added products. Anaerobic fermentation is a metabolic process regulated by a variety of functional microorganisms, whose diversity and dynamic changes are most influential factors[4]. Therefore, the comprehensive monitoring and deep analysis of microorganisms will improve the understanding of anaerobic fermentation for better stabilization of the fermentation system. However, traditional method was not able to monitor process completely due to the complexity and the quantity of microorganisms[5].

Denaturing Gradient Gel Electrophoresis (DGGE) is one of molecular biology method based on the different concentrations of denaturant in electrophoresis. In this method, the DNA double strands with the same length but different base sequence is dissociated at the corresponding concentration gradient. After the silver staining, DNA fragments with different sequence are separated on the gel with different strength[6-8]. Therefore, DGGE is considered a favor method to reveal the microbial community structure and monitor the microbial species dynamics[9].

Shi et al.[10] used DGGE to study microbial species and dominant species of anaerobic fermentation, and found that microbial species and dominant species had changed in different fermentation periods. Wang et al.[11] used DGGE to investigate the methanogenic archaea species in the low-temperature biogas digesters and analyze the changes of the dominant species before and after fermentation. The results showed that the dominant species of methanogenic archaea produced in different biogas samples differed from each other before and after low-temperature fermentation.

Two-phase anaerobic fermentation offers many advantages such as large organic load, high fermentation stability, short hydraulic retention time (HRT) and high gas production efficiency comparing with single-phase anaerobic fermentation[12,13]. Traditional anaerobic fermentation of livestock and poultry waste focus on high-concentration (TS is more than 9%), which generates other issues: high substrate viscosity and low mass transfer efficiency leading to incomplete fermentation. In this study, separated liquid (SL) separated using solid-liquid separator to significantly reduce its concentration. The separated solids were reutilized[14-19] and SL was disposed to produce biogas through two-phase anaerobic fermentation[20]. The microorganism population of two-phase anaerobic fermentation was investigate using DGGE, indicating the selectivity of microorganisms was separated when two-phase anaerobic fermentation of SL with the 6.6% of TS concentration. Finding in this study will fill the gap in literature and generate important knowledge leading to comprehensive utilization of dairy manure.

2 Materials and methods

2.1 Materials

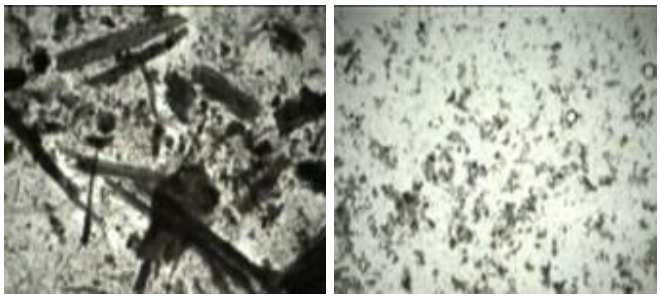
Dairy manure used in this study was provided by Wandashan

Received date: 2017-01-13 **Accepted date:** 2017-10-16

Biographies: Qiang Li, PhD candidate, research interests: biomass transformation and energy utilization, Email: lq4325356@163.com; **Guoxiang Zheng,** Professor, research interests: biomass transformation and microorganism utilization, Email: 99036951@qq.com.

*__Corresponding author: Zhengjun Guan,__ Professor, research interests: biomass transformation and energy utilization. Northeast Agricultural University, No.59 Mucai Street, Xiangfang District, Harbin 150030, China. Tel: +86-13351115923, Fax: +86 45155190608, Email: zhjguan@163.com.

Dairy Cattle Breeding Base of Harbin, China. Dairy manure was diluted with water at 2:1 mass ratio. SL was obtained by separating diluted dairy manure using a solid-liquid separator (DN140) invented by our laboratory. The inoculum was collected from an anaerobic continuous fermentation digester in our laboratory. The scanning electron microscopic (SEM) images of SL and diluted dairy manure (DDM) referring to non-separated diluted manure are shown in Figure 1. Table 1 summarizes the parameters of raw materials including TS, VS, lignocellulose, viscosity and carbon and nitrogen (C/N) ratio before anaerobic fermentation. SL shows significant lower lignocellulose content, viscosity and C/N ratio comparing to DDM with similar VS, indicating that SL provided better conditions for microorganisms activity than DDM.



a. DDM b. SL

Figure 1 Scanning electron microscopic image of DDM and SL

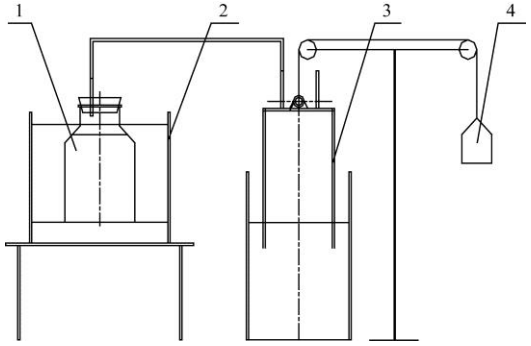
Table 1 Parameters of raw materials

	TS/%	VS/%	Lignocellulose*	Viscosity/mPa s	C/N ratio
DDM	5.79±0.12	4.86±0.21	57.66±0.87	237±23	40.12
SL	6.60±0.01	4.98±0.09	37.70±1.32	182±10	18.79
Inoculum	4.37±0.03	3.09±0.06	35.38±0.85	124±4	18.26

Note: * is % of TS.

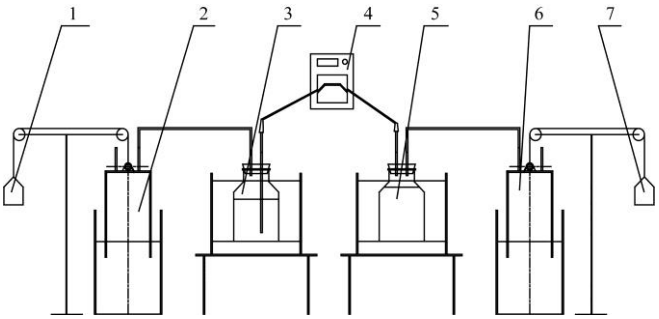
2.2 Experimental devices and conditions

Experimental devices of anaerobic fermentation are shown in Figures 2 and 3, respectively. The devices were composed of the temperature control part, the fermentation part and the gas gathering part. The volume of daily biogas generated was calculated based on the downward displacement of water. The effective volume of the fermentation tank was 4.5 L. Batch anaerobic fermentation was conducted at (5±2) ℃ for 20 d using 70% raw materials and 30% inoculations. Two-phase anaerobic fermentation experiment was carried out at medium temperature of (35±2) ℃. The TS of SL was 6.6%, the HRT of the acidogenic phase was 3 d, the HRT of the methanogenic phase were 6 d and 7 d, respectively, and subsequent fermentation 1 d. According to HRT of methanogenic, acidic liquid was transmitted quantitatively to the methane production tank of the start-up requirements using peristaltic pump[20].



1. Fermentation tank 2. Water bath 3. Gas gathering tank 4. Counter weight

Figure 2 Experimental device of batch anaerobic fermentation



1,7. Counter weight 2,6. Gas gathering tank 3. Acidification tank
4. Peristaltic pump 5. Methane production tank

Figure 3 Experimental device of two-phase anaerobic fermentation

2.3 Experimental methods

2.3.1 Anaerobic fermentation

In this study, the SL and DDM of batch anaerobic fermentation were evaluated firstly; afterwards the SL indicators of two-phase anaerobic fermentation were analyzed including the acidification characteristics, the start-up time of methanogenic phase, the different HRT calculated by the load of in-out materials, and the gas production performance. The microorganism population was analyzed by SEM, and DGGE. TS, VS and ash content were determined according to standard methods[21]. The pH was measured by HI9224 (Hanna Inc., Italy); the volume and composition of gas were measured by GC-6890N (Agilent Inc., USA); the total carbon was measured by VarioTOC (Elementar Inc., Germany); the total ammonia nitrogen and total nitrogen were measured by Kjeltec2300 (FOSS Inc., Denmark). All of the measurements were conducted in triplicate, and the averaged data are presented.

2.3.2 DGGE method

(1) DNA extraction

DNA extraction was carried out using Gao Pingping's method[22]. The kit (Ezup column genomic DNA extraction kit) was provided by Shanghai Biotechnology Company for purification.

(2) Amplification of variable region of 16SrDNA V3

16SrDNA V3 variable region bacterial specific primers:

P1:5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC ACGGGGGGCCTACGGGAGGCAGCAG-3', P2: 5'-ATTACCGCGGCTGCTGG-3'. The primers were supplied by Shanghai Biomedical Engineering Company.

The PCR reaction system consisted of 2.5 μL of 10 × PCR buffer (Mg^{2+} free), 2.0 μL $MgCl_2$ (25 mmol/L), and 2.0 μL dNTPs (2.5 mmol/L). There were 0.1 μL, 1 U Ex Tag enzymes in the upstream and downstream primers. DNA solution of 1 μL (1 mg) was filled up to 25 mL with distilled water. Reaction conditions: denaturation for 4 min at 94 ℃, degeneration for 30 s at 94 ℃, annealing for 30 s at 55 ℃, extension for 1 min at 72 ℃, cycling 30 times, finally extension to 5 min at 72 ℃. After the amplification products were electrophoreticly detected by 1% agarose gel, they went through 'Reconditioning PCR' to wipe off the heteroduplexes in PCR products[23,24].

(3) DGGE reconditioning

PCR products were separated by DGGE. The polyacrylamide gel with 8% concentration was converted to buffer solution whose gradients were 35%-60%, 60 ℃, 150 V, 1×TAE. The electrophoretic time was 14-16 h[25]. Avoid light dyed for 30 min by SYBR GREEN, and then rinsed them with deionized water. After staining, the samples were detected and photographed using

the ALPHA gel imaging system. The software of Quantity One was used to analyze the number of electrophoretic bands, the peak brightness and the mean optical density.

3 Results and Analysis

3.1 Comparison of SL with DDM in batch anaerobic fermentation

As shown in Figure 4, the peak of gas production of SL occurred earlier than DDM and the high gas period nearly ended on the 13th day. In contrast, the fermentation time of DDM was longer than SL resulting in more gas production at fermentation anaphase. The gas productions of SL and DDM at fermentation period (20 d) were 21.84 L and 28.74 L, respectively. The COD removal rate of SL was 65.40%, which was 16.3% higher than that of DDM. The VS methane yield of SL was 124.51 L/kg VS, 2.09 times than that of DDM (59.50 L/kg VS). COD and VS removal rate were calculated based on the influent and effluent COD and VS values (Table 2). These results indicated that SL has better fermentation performance than DDM.

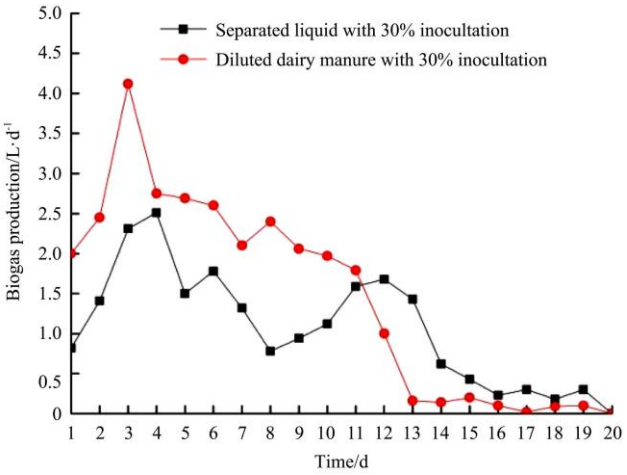


Figure 4 Comparison of daily gas production of DDM and SL in batch anaerobic fermentation

Table 2 Comparison of the anaerobic fermentation performance of DDM and SL

	COD removal rate /%	VS removal rate /%	Lignocellulose degradation rate/%	Methane content /%	TS methane yield /L (kg TS)$^{-1}$	VS methane yield /L (kg VS)$^{-1}$
DDM	49.10	35.19	33.57	45.90±0.90	49.94	59.50
SL	65.40	44.58	37.27	73.72±2.50	93.95	124.51

3.2 Microbial morphology characterization

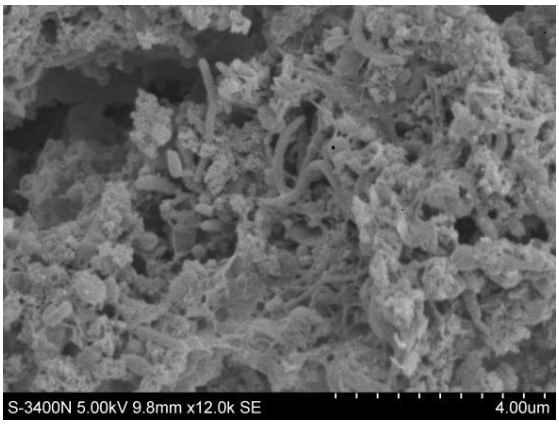
SEM was employed to analysis microorganism population during the fermentation process. Microbial morphology of two-phase anaerobic fermentation was observed in methaneogenic phase. The dominant populations of methanogenic phase were *Methanobacterium* and *Methanococcus*, such as *Methanobacterium sohngenii* and *Methanosarcina barkeri*. As acid concentration decreased, the growth rate of *Methanobacterium sohngenii* increased. In contrast, as the increase of acid concentration, the growth rate of *Methanobacterium sohngenii* tended to slow down, while the growth rate of *Methanosarcina barkeri* increased as acid concentration increased. In addition, its growth rate was very high. When acid was accumulated, *Methanosarcina barkeri* was able to easily become the dominant population of methanogenic. However, another study has found that the controlling of *Methanosarcina barkeri* growth was used to improving the efficiency of anaerobic fermentation, because *Methanobacterium sohngenii* has high affinity to acetic acid and formed particles sludge with better quality. When *Methanosarcina barkeri* became the dominant population, it has small shape and easily washed out from the reactor. According to the growth and variation rules of *Methanosarcina barkeri* and *Methanobacterium sohngenii*, the controlling of the acid concentration was able to yield lower growth rate of *Methanosarcina barkeri* than that of *Methanobacterium sohngenii*. High affinity of *Methanobacterium sohngenii* to substrate was used to develop the dominant population at the low substrate concentration, thus forming good granular sludge, which was beneficial for the methane production rate.

In Figure 5a, multiple microorganism population coexisted in the early stage, whereas *Methanococcus* was the main bacteria in the latter stage in Figure 5b. While in the methanogenic phase of SL, *Methanobacterium sohngenii* was the dominated microorganism. The images of microorganism and granular sludge are shown in Figure 6.

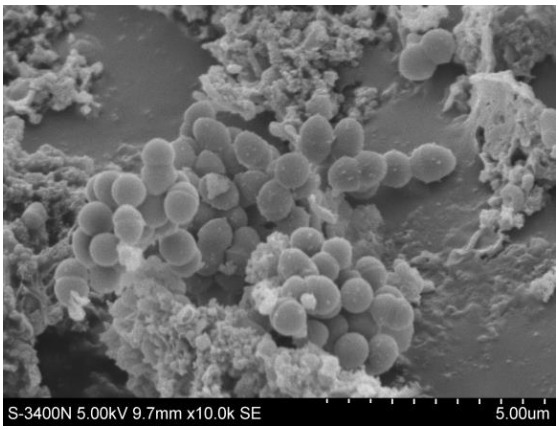
3.3 Analysis on two-phase anaerobic fermentation microorganisms

Fermentation microorganisms are a group of complicated bacteria, which require different hypoxia degree. They include organic decomposing bacteria and methanogenic bacteria. Organic decomposing bacteria is also known as non-methanogen bacteria, which includes fermentative bacteria, H_2-producing acetogens and acetic acid-producing bacteria; methanogen bacteria include hydrogen-eating methanogens and acetic acid-eating methanogens. These microorganisms play different transformation roles as nutritional needs. From complicated organic matter degradation to methane formation, the whole process is completed under their collaboration and interaction with each other.

In this study, microorganism population in the anaerobic fermentation system was dynamically tracked by DGGE. The results are shown in Figure 7. In DGGE map, the number of bands reflects the diversity of microbial species, while the banding intensity reflects the abundance of various biological species. Figure 7a shows the DGGE map of population during the start-up period. Lanes 1-3 represent main microbial between acidogen and methanogen microorganisms in synergistic process. Lanes 4-7 represent the DGGE map of the stage, in which the start-up process has ended and methanogens have become the dominant species. According to the distribution of the bands of the microbial species, the dominant microorganisms are concentrated clearly. The numbers of bands in the lanes are obviously reduced at only four bands, suggesting that two-phase anaerobic fermentation had achieved phase separation. Based on the comparison of bands' sequence (Tables 3 and 4) and the heredity relationship of different microbes in population (Figure 8), there were various microbial species in the acidogenic phase. The dominant microbial species were bacilli and cocci belonging to anaerobic and acid bacteria with higher activity. Meanwhile, there existed some *aerobacter aerogenes*. In Figure 7a, the similarity rates of the bands 8 and 14 are 100%, and the similarity rates of the bands 13 and 19 are 98%. They represent *Bacteroides* and *Veillonella* belonging to the anaerobic bacteria, and they took advantages of complicated organic matter to produce organic volatile acid. Most *Bacteroides*, such as *bacteroid* and *fusiformis*, belong to strict anaerobic bacteria with aerogenesis and acidogenic functions.

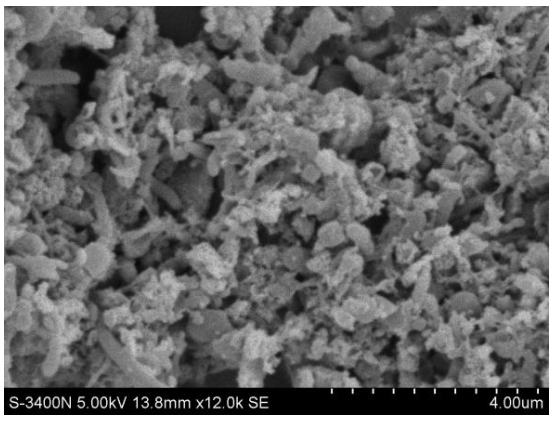


a. Early phase of batch fermentation

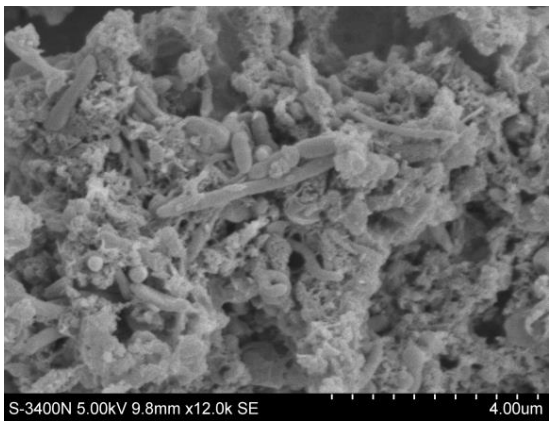


b. Middle phase of batch fermentation

Figrue 5 Microorganism images during the early and middle phases of batch fermentation by dairy manure

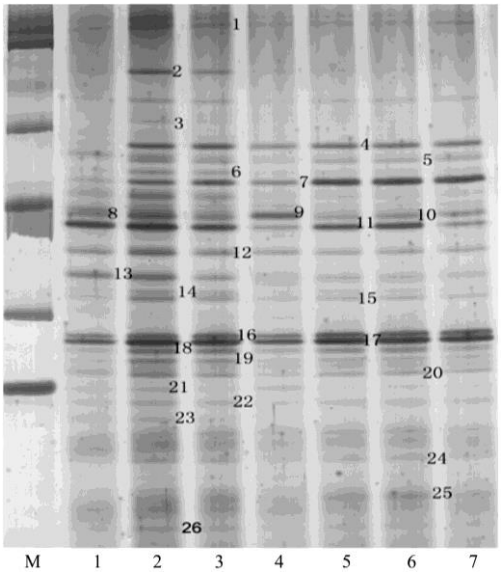


a. HRT 7 d

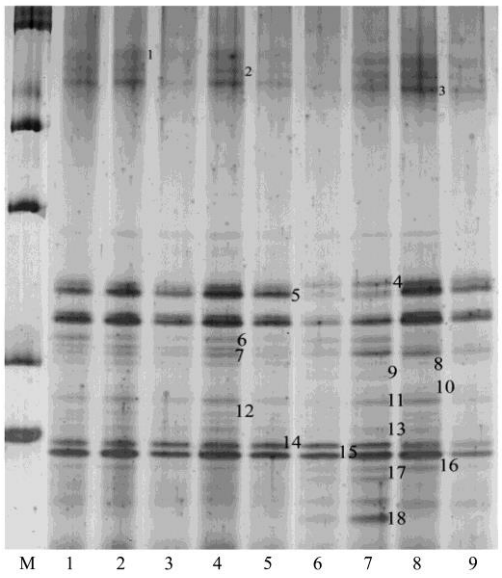


b. HRT 6 d

Figure 6 Methanogenic bacteria images at HRT 7 d and 6 d



a. In start-up period



b. In methanogenic phase at different loads

Note: M: mark-DL-2000; a. 1-7: start-up sample; b. 1-5: 1/7 load; 6-7: 1/8 load; 8-9: 1/5 load.

Figure 7 DGGE maps of bacteria in start-up period and in methanogenic phase at different loads

By monitoring the composition of microorganism species in the methanogenic phases, the number and composition of microorganisms species has changed significantly, as shown in Figure 7b. The number of bands is significantly reduced in lanes 1-9, indicating that the predominant species are 5, 14 and 15. These three bands represent *Enterobacteriaceae*, *Firmicutes* and *Corynebacterium*, respectively. The composition change of the species indicated that the fermentation conditions and the growth environment of the microorganisms had changed greatly from the acidogenic phase to the methanogenic phase. Microorganisms had to readapt to the methanogenic fermentation environment, resulting in the inhibition of the metabolic activity and the number of acidogen bacteria was significantly decreased. In contrast, after a period of adjustment, methanogen bacteria were domesticated in the methanogenic environment. The quantity and activity of acidogen bacteria were promoted to become the dominant species in the methanogenic phase. The bands 14 and 15 with methanogenic ability represent *Firmicutes* and *Corynebacterium*, respectively. The existence of these two types

of microorganism shows that *Firmicutes* and *Corynebacterium* as main methaneogen bacteria used dairy manure as raw material in this study. The results of SEM also confirm that there were a large number of bacillus and cocci in the acidogenic phase during the anerobic fermentation, and they were coordinated to maintain a relatively stable and balanced methanogenic system.

Table 3 Alignment results of different bands in DGGE profile during the start-up stage of methanogenic phase

Band number	Affinis bacterial strain	GenBank number	Similarity rate/%
1	Uncultured *Ruminococcaceae* bacterium	EU794262.1	100
2	Uncultured *rumen* bacterium	EU850483.1	92
3	Uncultured *Bacterioidetes* bacterium	GQ468585.1	96
4	*Flavobacteriaceae* str. SW072	AF493679.1	89
5	Uncultured *Lachnospiraceae* bacterium	EF708627.1	100
6	*Pseudomonas* sp. D3111	FJ161242.1	98
7	*Bacteroides finegoldii*	EU722740.1	92
8	Uncultured *cercozoan*	AB520719.1	100
9	*Chrvseobacterium* sp. HMD1043	GQ259742.1	100
10	Uncultured *Ruminococcaceae* bacterium	EU794305.1	96
11	*Clostridium caenicola*	AB221372.1	95
12	Uncultured *Bacteroidales* bacterium	EU573868.1	96
13	Uncultured *rumen* bacterium	GU304029.1	98
14	*Acinetobacter* sp. NFM2	GQ377756.1	100
15	*Pseudonocardiaceae* bacterium NBRC 105525	AB511316.1	99
16	*Bacteroides* sp. XB44A	AM230649.1	93
17	Uncultured *Firmicutes* bacterium	FJ651403.1	100
18	*Tissierella creatinophila* strain DSM 6911	GQ461823.1	97
19	Uncultured *Lachnospiraceae* bacterium	EF708603.1	98
20	Uncultured *Ruminococcaceae* bacterium	EU794234.1	98
21	*Bacteroides finegoldii*	EU722740.1	92
22	*Pseudoxanthomonas* sp.M967	AY368563.1	97
23	Uncultured *Ruminococcaceae* bacterium	EU794305.1	96
24	Uncultured *Bacterioidetes* bacterium	GQ468585.1	96
25	Uncultured *Bacterioidetes* bacterium	EU573868.1	96
26	*Tissierella creatinophila*	GQ461823.1	97

Table 4 Alignment results of different bands in DGGE profile during the operation stage of methanogenic phase

Band number	Affinis bacterial strain	GenBank number	Similarity rate/%
1	*Bacteroides finegoldii*	EU722740.1	93
2	Uncultured *Bacterioidetes* bacterium	AM157485.1	96
3	*Tissierella creatinophila* strain DSM 6911	GQ461823.1	97
4	*Enterococcus silesiaus* strain SS1792	GQ337036.1	96
5	*Acholeplasma parvum*	AY538170.1	89
6	*Leadbetterella byssophila* strain 4M15	AY854022.2	92
7	*Chrvseobacterium* sp. HMD1043	GQ259742.1	99
8	Iron-reducing bacterium	FJ269065.1	93
9	Uncultured *Ruminococcaceae* bacterium	EU794090.1	94
10	*Bacteroides* sp.	AB003390.1	96
11	*Brevibacillus agri* strain IHB B 1387	GU186123.1	99
12	*Bacteroides* sp.	EU834833.1	98
13	Uncultured *cyanobacterium*	EF106460.1	94
14	Uncultured *Firmicutes* bacterium	FJ651041.1	100
15	*Corynebacterium* sp. ICIRC105	GQ260084.1	100
16	*Phascolarctobacterium* sp. YIT 12068	AB490812.1	93
17	Uncultured *rumen* bacterium	GU303956.1	96
18	Uncultured *Bacteroidales* bacterium	EU794094.1	96

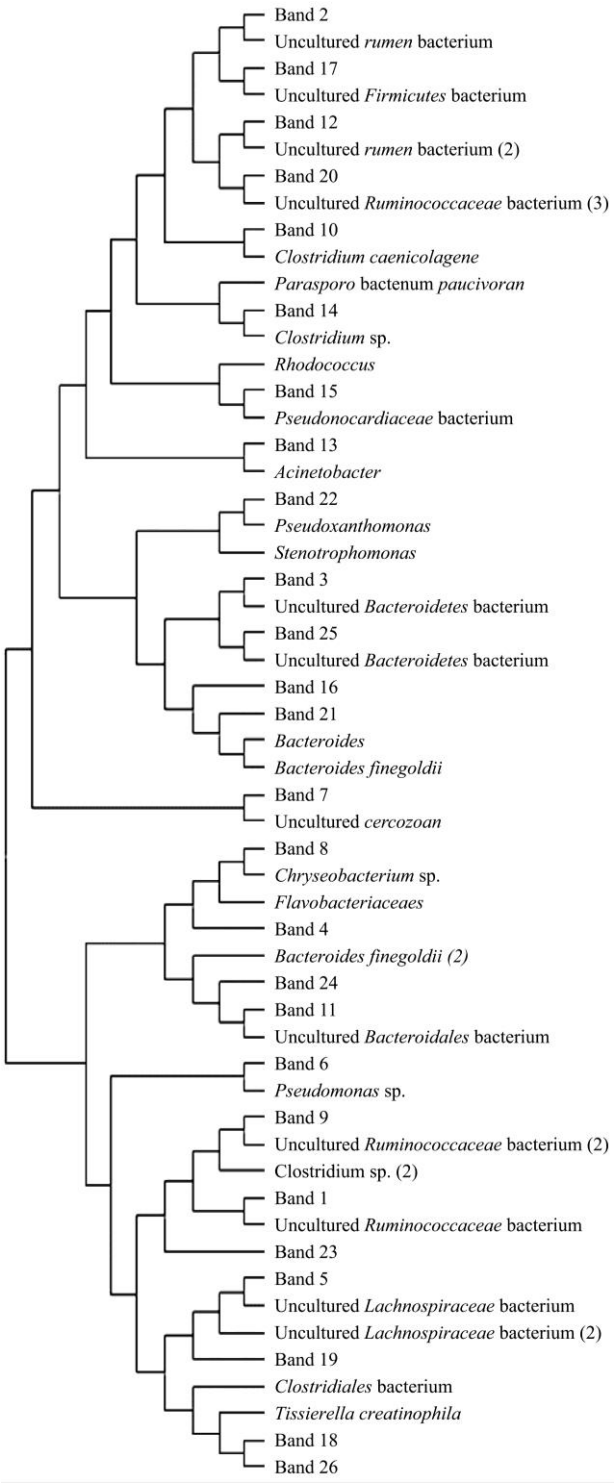


Figure 8 Phylogenetic tree of 16S rDNA excised from DGGE profiles during the start-up process of methanogenic phase

4 Conclusions

In batch anaerobic fermentation, the results showed that the lignocellulose content of SL was greatly reduced, the granule radius decreased significantly, and C/N was closed to 20:1. The COD removal rate of SL was 65.40%, which was 16.3% higher than that of DDM. The VS methane productivity of SL was 124.51 L/kg VS, which was 2.09 times than that of DDM (59.50 L/kg VS). Therefore, SL is a better feedstock for anaerobic fermentation than DDL.

The microorganism species were dynamically tracked by DGGE in acidogenic and methanogenic phases. The results

showed that both the *Bacteroides* (uncultured *Cercozoan* and *Acinetobacter* sp. NFM2, whose similarity rates were 100%) and the sibling species of *Veillonella* (uncultured rumen bacterium and Uncultured *Lachnospiraceae* bacterium, whose similarity rates were 98%) belong to strict anaerobic bacteria, which possess the function of aerogenesis and acidogenic. During the process of methaneogenic phase, *Firmicutes* (uncultured *Firmicutes* bacterium whose similarity rate was 100% and *Corynebacterium* sp. ICIRC105 whose similarity rate was 100%) had methanogenic function. The results also indicated that the two-phase anaerobic fermentation of SL had achieved phase separation. The acidogenic and methanogenic microorganisms became predominant population under the regulation of different ecological factors to decrease HRT and improve the efficiency gas production.

Acknowledgements

This study was supported by International Corporation Item (2013DFG62260) and Heilongjiang Province Natural Science Fund (E2015023).

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