Microbial Community and Population Dynamics Analysis of Anaerobic Fermentation of Vegetable Waste under Different pH

Hailing Ma, Mingyue Zheng, Mingxia Zheng, Guochen Zhang, and Kaijun Wang

***Abstract*—The advantage bacterium group of acidogenic and methanogenic processes by PCR-denaturing gradient gel electrophoresis (DGGE) under different pH values(4.1~4.3 and 5.9~6.1) was studied . 16S rDNA of bacteria and methanogenic archaea amplified with two universal primers (341F-GC /109F-GC and 518R). PCR amplicons were separated by denaturing gradient gel electrophoresis (DGGE) using the DGGE Decode system. Parts of the separated DNA were sequenced after purification from DGGE gel. The sequences of several 16S rDNA DGGE fragments were determined and some possible bacteria were confirmed in comparison in GeneBank (NCBI).The results showed that the acidogenic phase of fruits and vegetables during the fermentation process display different type: ethanol-type and butyrate-type fermentation and the predominant bacterium act differently. The predominant organism groups of methanogenic processes within the reactor performed differently, too. These results indicated that the change in the product formation was mainly caused by the change of the dominant microbial populations under different pH conditions.**

***Index Terms*—DGGE, different pH conditions, acidogenic phase, the predominant organism groups.**

I. Introduction

The pH values which have impacted on the microorganism community structure are important regulating factor of the digestion process [1]-[7]. By step shifting the culture pH in the acid reactor from 6.0 to 8.0, the main products were changed from butyric acid to acetic and propionic acids [1]. In a CFSTR, inoculated with activated sludge, the acidogenic dissimilation at 30℃ of a 1% glucose containing medium (C-limited), the maximum specific growth rate and product distribution, were studied over the pH range from 4.5 to 7.9, then it was found that pH value of 6.0 changed dramatically, from butyric acid to lactic acid and subsequently to acetic acid, formic acid and ethanol [2].

As modem molecular technology, PCR-DGGE [8], Fish [9], sscp [10] are powerful tools for the analysis of microbial communities, which can be used for studying the diversity and dynamics of microbial communities in many environmental samples.

The total DNA of microbes from anaerobic sludge in a digestion reactor of fruits and vegetables were isolated and analyzed by PCR-DGGE to study the bacteria diversity under different pH values.

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II. Materials and Methods

A. Activated Sludge Samples

In the study, a lab-scale reactor system was employed to treat fruits and vegetables wastes. This wastes were collected from Qinghe farmers markets at Beijing Haidian District and broken into smaller pieces to facilitate digestion with granular sludge which carry microorganisms. The characteristics of granular sludge during start-up were studied, as shown in Table I.

TABLE I: The Characteristics of Granular Sludge

	TS	VS	TSS	VSS
contents	9.51%	7.89%	9.48%	7.79%

The composition structure and diversity of microbial community of the granular sludge in the stabilization stage of the reactor were studied by molecular biology PCR-DGGE assessment. The study samples were shown in Table II.

TABLE II: The Sludge Samples in Different Reactor

Sample	origin
F0	raw sludge
A1	ethanol type fermentation in H1 reactor
A2	acid-producing type fermentation in H2 reactor
A3	methanogenic processes after ethanol type fermentation in the M1 reactor
A4	acidogenic processes after ethanol type fermentation in the M2 reactor
A5	acidogenic and methanogenic processes in the M3 reactor

B. Operation in Full-Scale Anaerobic Reactors

The effective volume of H1 and H2 are 1.5L, 500 grams of fruits and vegetables and 250 grams of granular sludge diluted with water to 1.5L and the PH values were adjusted to 4.2and 6.0 by PH value controller at reactors startup, respectively.

The effective volume of M1 and M2 are 4.0L, 160 grams of fruits and vegetables (Sodium bicarbonate solution ere used for altering the pH values to 5.96) and 2000 grams of granular sludge diluted with water to 4.0L.

The effective volume of M3 is 5.0L, 200 grams of fruits and vegetables (Sodium bicarbonate solution are used for altering the pH values to 5.96) and 2500 grams of granular

sludge diluted with water to 5.0L.

All these five reactors work with artificial feeding after let it set for two days. The parameters of operation are shown in Table III below:

TABLE III: OPERATION PARAMETERS OF REACTORS

parameters	two-phase(ethanol-type fermentation)		two-phase(butyrate-type fermentation)		one-phase
	H1	M1	H2	M2	M3
HRT (d)	3	12	3	12	15
The effective volume (L)	1.5	4.0	1.5	4.0	5.0
feeding rate (mL d-1)	500	333	500	333	333
feed concentration (gVS L-1)	40.8	/	40.8	/	40.8
organic load (gVS (L d)-1)	13.6	/	13.6	/	2.7

C. DNA Extraction from Samples

Aportion of 0.5g of mixed granular sludge from each treatment were subjected to genomic DNA extraction according to instruction of test kits (FastDNA® Spin Kit for Soil) and conducted DNA quantification and purity analysis. DNA fragmentation was detected by agarose gel electrophoresis.

D. Reference Strains and PCR Amplification

The PCR was performed in a final volume of 50μL containing 5μL of DNA buffer ,4μL of dATP, 0.8μL of each primer ,8μL of extracted DNA solutions and 30.8μL sterile water.

TheprimerpairGC-341f(5'-CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3')and518r(5'-ATTACCGCGGCTGCTGG-3')wasusedforamplifyingeubacteriaDNA,whereasprimerpairGC-109f(5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACKGCTCAGTAACAC GT-3') [11] and 518r was used for the methanogens from granular sludge. The primers (341f and 518r) were used by Muyzer G [12].

For eubacteria, PCR amplification was performed in a 96-multiwell GeneAmp PCR system 9700 (Applied Biosystem) with initial denaturation at 94℃ for 5 min, followed by 19 cycles of denaturation at 94℃ for 1 min, annealing at 64℃ for 1 min elongation at 72℃ for 1 min and followed by 4 cycles of annealing at 64℃.

For methanogens, PCR amplification was performed with initial denaturation at 94℃ for 5 min, followed by 34 cycles of denaturation at 94℃ for 1 min, annealing at 57℃ for 1 min elongation at 72℃ for 1 min and elongation at 72℃ for 10 min.

PCR amplicons were used for sequence-specific separation by DGGE according to Zoetendal *et al.* [13] using a Dcode DGGE System (Bio-Rad, USA). For primer pair GC -341f/518r and GC-109f/518r, DGGE was performed in 8% (w/v) polyacrylamide gels (acrylamide/N,N'-methylene bisacrylamide ratio, 37:1 [w/w]) in 1.0×TAE buffer. The denaturant gradient range of the gel, in which 100% denaturant contained 7 mol l^{-1}urea and 40% (v/v) formamide, was 30–60%and 30–70% respectively.

The electrophoresis was initiated by prerunning for 10 min at 20V and subsequently ran at 70 V (eubacteria and 60V (archaebacteria.) for 16 h at 60C. The gel was stained with 0.01% GelRed for 30min according to HongWeiShi etal. [14] and scanned using gel imaging and analysis system (Bio-Rad, USA). The DGGE profile was analyzed by Quantity One software (Bio-Rad).

Nucleotide sequences of DNA fragments recovered from bands on DGGE gels were determined by the following method. Interesting bands on DGGE gels were excised with a 1ml pipet tip into a 1.5ml tube and the DNA was eluted in 30μl TE at 4°C overnight. The primer pair without GC clamp (341F or 109F and 518R) was used in the template amplification by PCR for the subsequent cycle sequencing. And the amplified products were analyzed by agarose gel electrophoresis and DNA sequencing by BGI.

III. RESULTS

A. Run Results

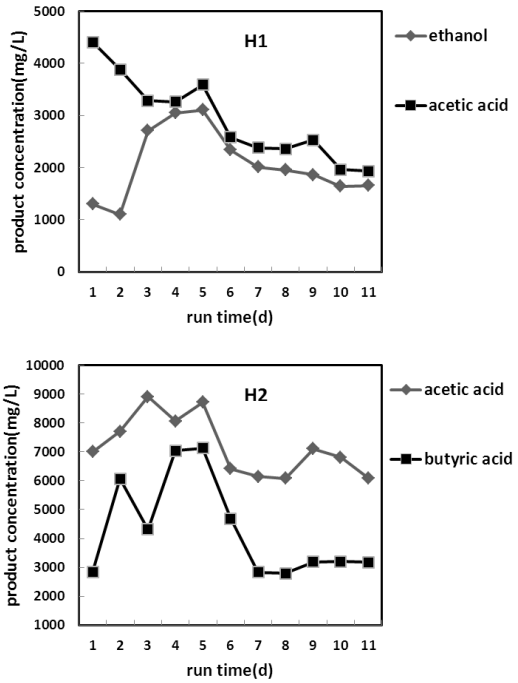


Fig. 1. The change of products of ethanol type fermentation and butyrate-type fermentation.

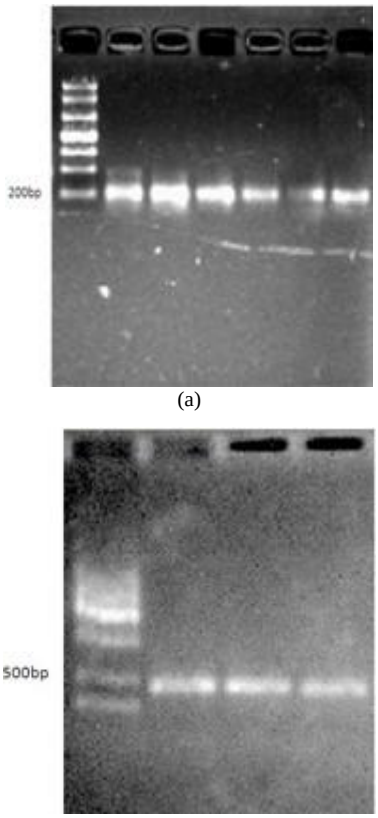
Fig. 1 shows the products in the acid reactor during the pH shift at 4.1-4.3 (a) and 5.9-6.1 (b). As shown in Fig. 1(a), the main products at pH 4.1-4.3 were ethanol and acetic acid, while the butyric acid and propionic acid concentrations were rather low, Whereas the culture pH in the acid reactor from 5.9 to 6.1, the main products changed from ethanol butyric acid to butyric acid as shown in Fig. 1(b).

From the dynamic changes of products, we can see there was significant ethanol type fermentation with H1 and butyrate-type fermentation with H2, as shown in Fig. 2. It is of great significance for guaranteeing fast and steady continuous-running reactor to alter the pH level.

B. PCR Amplification of DNA

As shown in Fig. 2, the PCR products from primer pairs 109f/341rGC, 109fGC/518r were strong for the different samples as mentioned in Table II. The primer pairs 109f／341rGC amplified around the hypervariable V3 region of

the 16S rRNA gene, giving a PCR product of about 200 bp. And Primer pairs109f /518r generated clear PCRproducts Archaea samples with 500 base pairs. A clear single target band indicates that the obtained DNA fragments with high specificity can be used for further experiments.

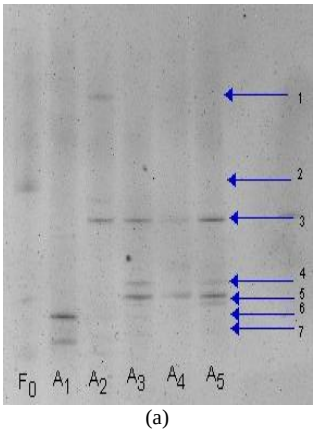


(a)

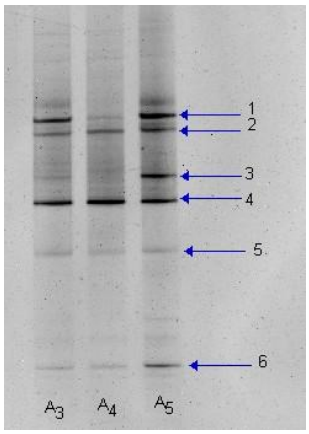
(b)

Fig. 2. Agarose gel electrophoresis of PCR amplified 16SrRNA(V3) gene of bacteria (a)and archaea(b).

The PCR products were run in DGGE to reveal the dominant microorganisms present in the samples. According to DGGE fingerprinting, significant variance between the different samples were observed (Fig. 3). Two especial bands (band 6 and band 7) in the A1 (Fig. 3a) were found, which were ethanol type fermentation in acidogenic phase with pH value at 4.1-4.3. Band 1 and band 3 in the A2 (Fig. 3a) were observed from the granular sludge in acidogenic phase of butyrate-type fermentation with pH value at 5.9-6.1. However, the band 3corresponding to such species was present in all the methanogenic processes (Fig. 3a, A2, A3, A4，A5),which means the band 3 has the strong capacity of acid material production .



(a)



(b)

Fig. 3. DGGE analysis of the 6 activated sludge samples, listed in Table II. Bacteria (a); Archaea (b).

For archaea, band patterns on DGGE gels with primer pairs 109f-GC and 518R showed good resolution and separation (Fig. 3b). Changes in the community structure of A3, A4 and A5 were shown Fig. 3b of DGGE fingerprinting from methanogenic processes after ethanol type fermentation in the M1 reactor, acidogenic processes after ethanol type fermentation in the M2 reactor and acidogenic and methanogenic processes in the M3 reactor. The number of DGGE bands of A4 was less than A4's. Microorganism might be inhibited by butyric acid on some level. And the number of DGGE bands of A3 was almost equal to the number of A5 in the uniphase reactor. It's possible that because the methanobacteria has been less affected by the alcoholated materials.

Four amplified bacterial bands (X3,X4, X5, X6) and five archaea bands (g1, g2, g3, g4, g5) as marked on Fig. 3 were selected for DNA sequencing, respectively. Sequences of the DGGE bands were compared to those present in the databases using BLAST search program at the NCBI web site. Phylogenetic trees were constructed by 1000-fold bootstrap analysis using the neighbor-joining method with MEGA4.0, as shown in Fig. 4 and Fig. 5.

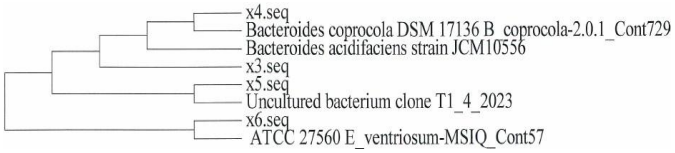


Fig. 4. the phylogeny trees of bacteria during anaerobic digestion of treating fruits and vegetables wastes.

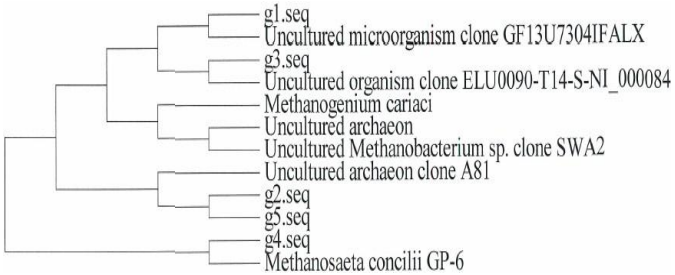


Fig. 5. The phylogeny trees of archaea during anaerobic digestion of treating fruits and vegetables wastes.

For bacteria, band 6 is the special strain during the ethanol type fermentation in this study. After NCBI blast, no homologous strain (lower identity than 83%) was found. It may imply that this strain is probably novel one. We will conduct a study on ethanol type fermentation communities

under single phase situation by other biotechnological methods presented here to assess the strain in a future paper. However, the comparability of the band 3 sequences exists in all methanogenic processes (M1, M2, M3) with acteroides acidifaciens strain JCM10556 is 97%. It indicated that acteroides acidifaciens strain JCM10556 producing acids is the dominant strain during methanogenic processes.

Representatives of archaea bands that were clear were excised from DGGE gels as many as possible and subjected to sequencing. The phylogenetic tree of the 5 sequenced bands is shown in Fig. 5. All fragments belonged to methanogenic archaea, which is consistent with theoretical conclusion of Shi *et al.* [14] which showed that Bacteroidetes, Methanosaeta and Methanospirillum like clones dominated in fruits and vegetables wastes reactor soils under single phase situation by analyzing clone libraries of archaeal 16S rDNA. g4 was affiliated to Methanosaeta which was discovered in M1, M2 and M3 mentioned in Table II.

IV. Discussion

The method for DGGE analysis of 16S rDNA described above is suitable for investigating bacteria and methanogenic archaeal community of granular sludge in single phase and two-phase anaerobic sludge digestion reactors. It was considered that the change in the product formation was mainly caused by the change of the dominant microbial populations under different pH conditions.

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