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Full Length Research Paper

Microbial diversity in a full-scale anaerobic reactor treating high concentration organic cassava wastewater

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Microbial characteristics in the up-flow anaerobic sludge blanket reactor (UASB) of a full-scale high concentration cassava alcohol wastewater plant capable of anaerobic hydrocarbon removal were analyzed using cultivation-independent molecular methods. Forty-five bacterial operational taxonomic units (OTUs) and 24 archaeal OTUs were identified by building 16S rRNA gene of bacterial and archaeal clone libraries. Most bacterial OTUs were identified as phyla of Firmicutes (53.3%), Chloroflexi (20.0%), Proteobacteria (11.1%), Bacteroidetes (6.7%) and a candidate division (2.2%). *Methanosaeta* (57.5%) were the most abundant archaeal group, followed by *Methanobacterium* (10.6%), *Methanomethylovorans* (8.5%) and *Methanosarcina* (6.4%). Most bacterial species take charge of cellulolysis, proteolysis, acidogenesis and homo-acetogenesis; the most methanogens were typical hydrogenotrophic or hydrogenotrophic/aceticlastic. This study revealed a succession of both bacterial and archaeal populations during the trial, which could be linked to operational adaptation of high concentration organic cassava wastewater.

Keywords: Full-scale, anaerobic reactor, 16S rRNA gene clone library, microbial diversity, functional analysis.

INTRODUCTION

Fuel ethanol production from cassava in China has grown rapidly due to the increasing demand for renewable resources. However, the large quantity of wastewater with high concentrations of organic pollutants produced from ethanol fermentation has been an intractable problem in larger scale bioethanol production. The production of 1 t of bioethanol results in 9 to 15 t of distillery wastewater. Cassava wastewater has a very high chemical oxygen demand (COD) of 20 000 to 30 000 mg/L and a low pH of between 3 and 4, especially in fresh crop season. The disposal costs of wastewater resulted in a large burden to the cassava based ethanol industry, and the environmental problems cannot be neglected (Thammanoon et al., 2010). Therefore, it is urgent to devise a new strategy for treatment of the organic wastewater.

Anaerobic biological treatment of high concentration organic wastewater is a proven technology that has been widely applied. COD removal efficiencies of more than 90% and volumetric loading rates of up to 16 kg COD/m^3·d, and even higher, is possible. However, there is a concern that biological treatment systems often do not perform well on campaigning industries and long start-up periods. A novel full-scale anaerobic fermentation process with 1600 m^3 volume was proposed to treat high concentration cassava wastewater resulting after alcohol distillation. The organic loading rate may be as high as 20 kg COD/m^3·d in fresh crop season. Such high efficiency of organic matter degradation and biogas production capacity in cassava wastewater treatment industry has not been reported. However, the operating mechanism and the characteristics of activated sludge microbes were not clear, especially when loading high concentration organic wastewater.

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Abbreviations: UASB, Up-flow anaerobic sludge blanket reactor; **OTUs,** operational taxonomic units.

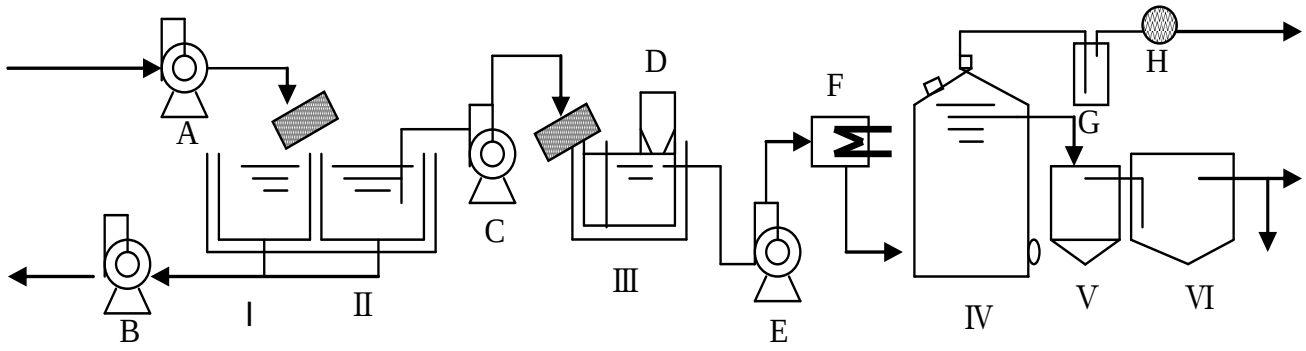

Figure 1. Flow chart of full-scale anaerobic process. (I) Primary sedimentation pond; (II) pretreatment pond; (III) adjusting pond; (IV) UASB reactor; (V) recycling tank; (VI) secondly sedimentation pond; (A) wastewater pump; (B) slag pump; (C) influent pump in adjusting pond; (D) adjusting system; (E) influent pump of UASB reactor; (F) warming system; (G) water sealed slot; (H) gas flow meter.

In recent years, some studies have depicted the microbial community structures of anaerobic digester sludge using a 16S rRNA gene approach in order to take full advantage of renewable biomass through anaerobic digestion technology. One of the most advanced fields associated with the technology in the past few years is the microbiology of anaerobic digestion processes (Godon et al., 1997; Chouari et al., 2005; Herto et al., 2007; Michael et al., 2007). The ecology and function of the microbial community in these processes are required to control the biological processes for operational efficiency (Tomoyuki et al., 2006; Chen and Cheng, 2008). Culture-independent, biomolecular methods have the potential to make a valuable contribution and new information has recently been obtained (Cheon et al., 2008; Liu et al., 2010), using molecular approaches targeting the 16S rRNA gene, such as PCR-DGGE and genomic cloning.

For the purpose of both basic research and biogas biotechnology, there is considerable interest in elucidating the microbial composition and metabolic diversity, as well as setting up an applied molecular fingerprint monitor method involved in biogas production. In the present work, we described bacterial and archaeal diversity of a full-scale anaerobic bioreactor using several molecular techniques such as 16S rRNA gene library construction, revealing the metabolism of major microbial species.

MATERIALS AND METHODS

Sludge samples

Sludge samples were collected from a real full-scale anaerobic digester plant treating cassava ethanol wastewater in Ming Yang (Guangxi, China). The flow chart of the studied plant used in this work is shown in Figure 1. The reactor temperature was maintained between 30 and 35°C, the design COD (chemical oxygen demand) loading was 20 kg and the pH was maintained around 7.0 during the operation. When sampling, the reactors were operating efficiently with the COD removal rate over 80%. All of the samples were collected in sterile bottles and processed in the laboratory within 24 h after sampling.

Water quality analysis

For evaluation of the stability and performance of the plant, chemical oxygen demand (COD), ammonia nitrogen (NH_4^+-N), total nitrogen (TN), total phosphorus (TP) and acetic acid of influent and effluent from the plant were measured as standard methods (AOAC,1990).

Genomic DNA extraction

Two milliliter sludge samples were washed three times using phosphate-buffered saline (PBS, pH 7.0) and centrifugated at 4°C, 10,000 rpm, for 15 min. The genomic DNA was then extracted using a benzyl chloride method (Zhu et al., 1993).

Analysis of 16S rRNA gene clone library and phylogenesis

Amplification of 16S rRNA genes from purified genomic DNA was carried out using primer B27f-B907r for the bacterial community and A109f-A912rt for the archaeal community. The bacterial PCR was denatured for 10 min at 95°C. A total of 25 cycles, each including 1 min at 93°C, 1 min at 50°C, and 1.5 min at 72°C, was followed by a final extension step of 5 min at 72°C. The thermal profile of archaeal PCR for clone library analysis was as described earlier, except that 30 cycles were applied for amplification (Hori et al., 2006; Wang et al., 2011). The products were examined by electrophoresis on 2% agarose gel before being subjected to further analysis. Then the products were ligated into pGEM-T Easy Vector (Promega, USA) according to the manufacturer's protocol after purification by using the TIANgel Midi Purification Kit (Tiangen, China). All white colonies were randomly picked and screened by DGGE profile as described above. A total of 120 white clones that produced a single band with different melting positions were selected for sequence analysis.

The insert DNA fragments were sequenced at SunBiotech Developing Center (Beijing, China). Sequence similarity searches were performed in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) using the blast database. The tree was constructed by using CLUSTAL X software program and MEGA 4.0 software, referring neighbor-joining method. The sequences generated by this study were deposited in the NCBI GenBank database under the accession numbers JN596353- JN596421. All clones having a

Table 1. Mean treatment results for various parameters of water quality by the plant.

Parameter	Concentration		Mean removal rate (%)
	Influent	Effluent	
pH	3.4 ± 0.5	7.0 ± 0.3	-
SCOD (mg/L)	30180 ± 150	448 ± 30	98.5
TN (mg/L)	3.32 ± 0.10	0.95 ± 0.01	71.4
TP (mg/L)	21.81 ± 0.10	8.25 ± 0.10	62.1
Acetic acid (mg/L)	2.09 ± 0.02	0	100

sequence similarity of more than 97% with each other were grouped into one OTU (Schauer et al., 2005).

Denaturing gradient gel electrophoresis (DGGE)

Colonies containing 16S rRNA gene inserts were screened by DGGE analysis (Gonzalez et al., 2003). The clones that produced a single DGGE band with different melting positions were selected for sequence analysis. The primers for bacterial 16S rRNA gene PCR amplification were 357F-GC (5'-CCTACGGGAGGCAGCAG-3')with a GC-clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') and 517R (5'-ATTACCGCGGCTGCTGG-3'). The archaeal primer set A348If (5'-GGIGCAICAGGCGCGAAA-3') and U806Ir (5'-GGACTACCIGGGTITCTAA-3') with the GC clamp (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCC-3') for PCR to amplify the V3 region of 16S rRNA gene (Hori et al., 2006). The PCR-DGGE was carried out using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA) with some modifications. The denaturing gradient was 20 to 60% (where 100% was defined as 7 M urea with 40% formamide). Polyacrylamide gels were stained with SYBR Green I (Molecular Probes, Eugene, OR) for 30 min (Hori et al., 2006). Clones that pro-duced a single band with different melting positions were selected for sequence analysis.

RESULTS

Performance of the full-scale anaerobic plant

When sampling, the effluent COD reduced to 448 mg/L when the influent COD concentration was 30 180 mg/L, the highest efficiency of which was 98.5%. The mineralization rate of total organic nitrogen was 94.8%, falling to 0.05 g/L; the conversion rate of total phosphorus content was 62.1%, reducing to 8.25 mg/L; the concentration of acetic acid dropped to zero from 2.09 mg/L (Table 1).

Analysis of clone library

All of the white clones of two clone libraries were screened by DGGE. Bacterial library (BL) and archaeal library (AL) of 16S rRNA gene comprised of 65 bacterial clones and 55 archaeal clones, while 45 bacterial OTUs and 24 archaeal OTUs were identified. The clones that produced a single DGGE amplicon with a melting position identical to that of one of the dominant bands clone library DNA patterns were selected for sequence analysis (Figure 2).

Diversity of bacterial community

Except three unidentified phylotypes, the distribution of all bacterial clones was into five major phyla as follows: *Firmicutes*, *Chloroflexi*, *Proteobacteria*, *Bacteroidetes* and a candidate division (undetermined taxonomic status), the proportions of which were 53.3, 20.0, 11.1, 6.7 and 2.2%, respectively (Figure 3). In addition, there were 4.4% unidentified anaerobic bacteria, and 2.2% of an undetermined toluene-degrading methanogenic consortium of bacteria. The *Firmicutes* bacteria were, by far, the dominant group. Species from these five phyla were likely ubiquitous in most anaerobic digester as nearly all studies have found (Liu et al., 2010; Herto et al., 2007).

Diversity of archaeal community

All of archaeal clone sequences were classified within the phylum *Euryarchaeota*. The similar strains investigated were classified into 24 OTUs (Figure 4). The majority of these related species were attributed to four genera: *Methanosaeta*, *Methanosarcina*, *Methanobacterium* and *Methanomethylovorans*, the proportions of which were 57.5, 6.4, 10.6 and 8.5%, respectively. Meanwhile, there were also 6 OTUs (17.0% of AL) that could not be found with the similar category in NCBI database. Based on the phylogenetic relationship in Figure 3, these ones were classified as three clusters. The unclassified cluster I and unclassified cluster II both contained 1 OTU (4.3 and 2.2% of AL). The 4 OTUs (10.6% of AL), including C3, C8, C16 and C29, could be phylogenetically merged as one cluster named unclassified cluster III. However, the defined taxonomic level of three unclassified clusters had not been so clear.

Metabolic functions of bacterial communities

The most dominant *Firmicutes* group could further be divided into five groups, Clostridiaceae (24.4% of BL),

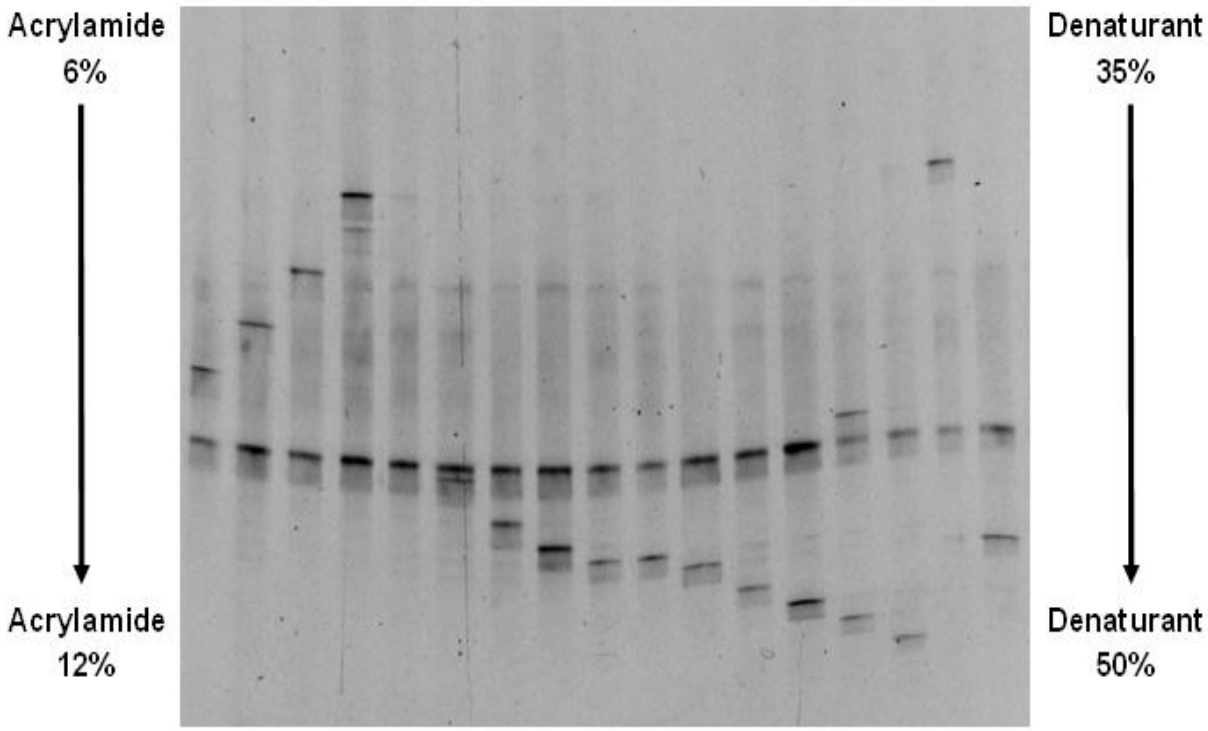

Figure 2. DGGE profile for clone screening. A single DGGE amplicon with a melting position identical to that of one of the dominant bands.

Eubacteriaceae (13.3% of BL), Planococcaceae (6.7% of BL), Peptococcaceae (4.4% of BL) and Lachnospiraceae (3.1% of BL). The vast majority of strains in this group might hydrolyze glucose, proteins, casein, cellulose, and pyruvate into small molecular organic acids: acetate, propionic acid and others, Meanwhile, some strains such as: *Levilinea saccharolytica*, were also able to use H_2/CO_2 to synthesize acetate, while others such as: *Longilinea arvoryzae*, could ferment acetate to produce hydrogen (Zellner, 1996; Lee et al., 2007; Imachi et al., 2007; Worm et al., 2009). The *Chloroflexi* group included 9 OTUs. *L. saccharolytica* might use H_2/CO_2, propionic acid to produce acetate. The growth of a *Bellilinea caldifistulae* strain and a *L. arvoryzae* strain were both enhanced in co-cultivation with hydrogenotrophic methanogens when yeast extract was required (Zellner, 1996; Yamada et al., 2007).

The *Proteobacteria* clones affiliated to *Moraxellaceae* and *Syntrophaceae*. *Acinetobacter lwoffii* could denitrify the sludge material into small molecules, forming NH_3-N. It also could degrade lignin (Ku et al., 2000). The clones of *Syntrophaceae* coexisted with hydrogen-utilizing microbes, to degrade fatty acid into acetate and H_2 (Gray et al., 2011). The *Bacteroidetes* clones all belonged to *Prevotellaceae*. The strains played a significant role in the metabolism of proteins, peptides and starch. They might utilize water soluble cellodextrins and some strains had considerable carboxymethyl-cellulase (CMCase) activity (Gardner et al., 1995).

Metabolic functions of archaeal community

In our study, the *Methanosaeta* and *Methanosarcina* groups accounted for 63.8% of the clone library together. The *Methanosaeta* clones could be divided into 10 OTUs (57.5% of AL), and the *Methanosarcina* into 2 OTUs (6.4% of AL). It was found that the specific filamentous fiber formed by *Methanosaeta* could provide network structure connecting other microbes to shape biofilm, which played an important role in granular sludge formation, especially in high loading and complex components organic wastewater treatment process (Lee et al., 2008). These two groups were in competition, but also in a mutually beneficial symbiosis, together constituting the major source of anaerobic methane production (Calli et al., 2005).

The *Methanobacterium* group contained 3 OTUs (10.6% of AL). Some strains were hydrogen-utilizing type or molecular nitrogen-utilizing type. There were also some strains needing special substrate for metabolism (Schauer and Ferry, 1980; Magingo et al., 1991; Ma et al., 2005). These two *Methanomethylovorans* phylotypes described were all enriched on either trimethylamine or dimethyl sulfide. The only substrates utilized by *Methanomethylovorans hollandica* strain DMS1^T were methanol,

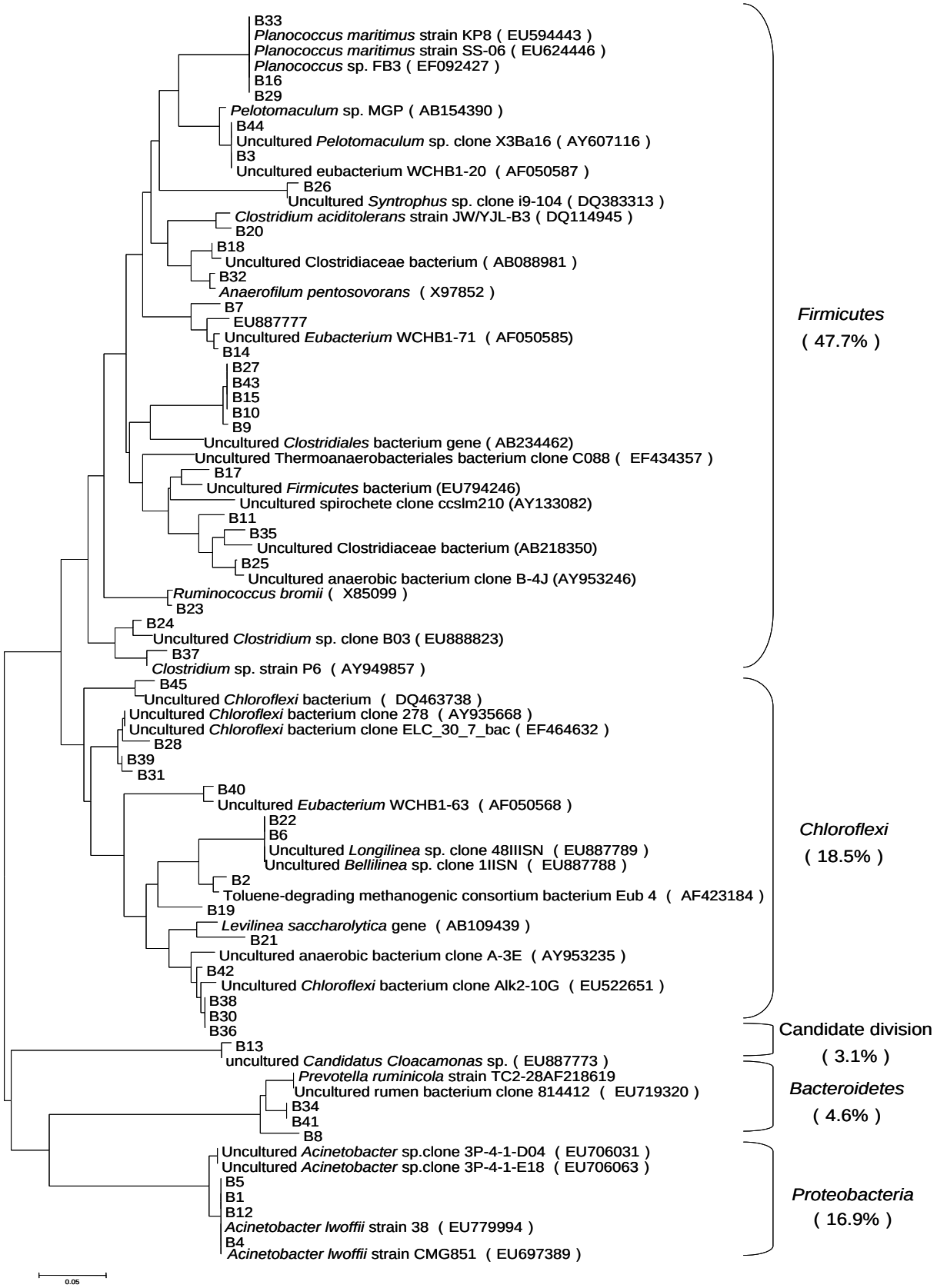

Figure 3. Phylogenetic analysis results of the bacterial OTUs retrieved from the bioreactor samples. Evolutionary dendrogram constructed using the NJ method. Scale bar denotes 0.05 indicated changes per nucleotide.

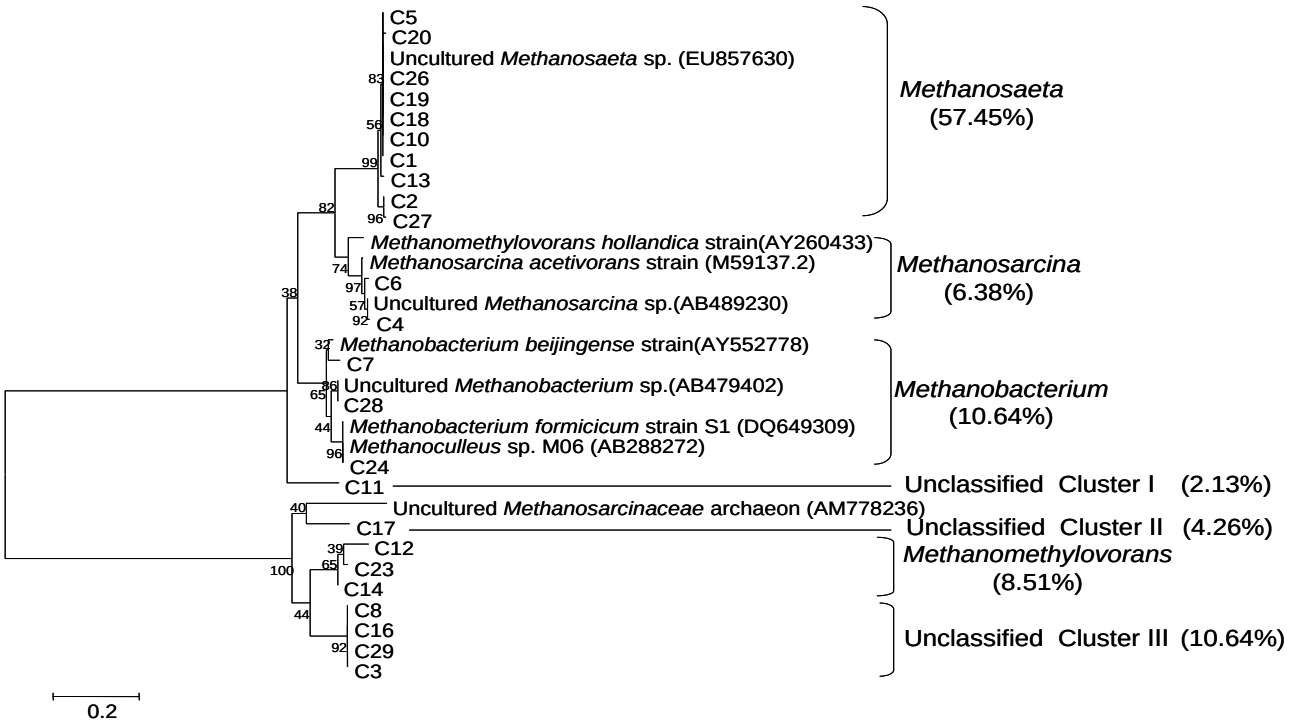

Figure 4. Phylogenetic analysis results of the archaeal OTUs retrieved from the bioreactor samples. Evolutionary dendrogram constructed using the NJ method. Scale bar denotes 0.2 indicated changes per nucleotide.

methylamines, methanethiol, and dimethyl sulfide (Lomans et al., 1999).

DISCUSSION

Anaerobic digesters are widely used to treat different wastes, example brewery and pulp industry wastewater containing different carbohydrates, long-chain fatty acids, volatile fatty acids, methanethiol and terephthalate. In this study, stable, efficient bioreactor performance was achieved in a full-scale cassava ethanol distillery wastewater plant, as demonstrated by 98.5% COD removal efficiency, stable effluent pH value at about 7.0, total organic nitrogen mineralization rate of 94.8% and total phosphorus conversion rate of 62.1%. The continued and consistent stability achieved in organic processing wastewater plant highlight the case for potential full-scale application of this treatment regime.

Most of the microbial groups play a special role in the final product formation in succession. Various types of bacteria could produce substrates for methanogens. Sequence analysis suggested that several similar bacterial strains were affiliated with carbohydrate utilizing types like the Eubacteriaceae, Planococcaceae, Anaerolineaceae, or some members of Clostridiaceae. Clones related to strains using amino acids (example Peptococcaceae and Prevotellaceae) or short branched fatty acids (example Syntrophaceae) were detected, except that some strains were assigned to potential cellulose utilizing like *Prevotella ruminicola* and *Acinetobacter lwoffii*. Thanks for the organic molecules hydrolysis, acetate, ethanol, H_2/CO_2 and other volatile organic acid were accumulated, all of which are the substrate just for the growth of methanogens. For archaeal community, molecular analysis revealed the presence of H_2/CO_2/formate-oxidizing *Methanobacterium*, the H_2/CO_2-oxidizing *Methanosarcina*, the acetate-splitting *Methanosaeta* or the formate-oxidizing *Methanomethylovorans*. These finding are in agreement with the common hypothesis that methane is normally produced by hydrogenotrophic and aceticlastic archaea, a few by methylotroph archaea (Sekiguchi et al., 1998). Similar results have been found in some other distillation industry wastewater treatments, such as: winery, brewery and peach-lye canning effluents.

Cloning and sequencing of 16S rRNA gene has been frequently applied to elucidate the exact composition and taxonomic category of a microbial community. However, there were still some microbial clones very hard to be classified into a phylogenetic cluster (6.6% of BL, 17.0% of AL), so it may be speculated that new microorganisms were present. At the same time, most of the bacterial clones (67.7% of BL) and archaeal clones (81.8 % of AL) were closely related to uncultured strains. Interestingly, there was also a candidate division WWE1 in bacterial clone library, which was always abounded in anaerobic sludge, but their physiological metabolism had not been

known. These results therefore demonstrated the importance of culture-independent molecular methods for the study of wastewater communities.

There must be some errors in detecting the micro-organism community according to the construction of the gene library. Because the different cells have different 16S rRNA gene copies, non-suitable primer sets can lead to a discrimination of certain microbial species. In this study, many modified methods were utilized to try to reduce errors, such as, benzyl chloride method utilization for genomic DNA extraction which had been universal application and multiple PCR reaction system (Martin et al., 2001). Meanwhile, in order to guarantee the cloning library coverage, DGGE technology was used in clone library construction. The clones for sequencing were finally picked out according to the different bands location in DGGE profiles. This approach was also cost-effective.

ACKNOWLEDGEMENT

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