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Diversity and Distribution of Methanogenic Archaea in an Anaerobic Baffled Reactor (ABR) Treating Sugar Refinery Wastewater

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The diversity and distribution of methanogenic archaea in a four-compartment anaerobic baffled reactor (ABR) treating sugar refinery wastewater were investigated by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). At an organic loading rate of 5.33 kg COD/m^3·day, the ABR could perform steadily with the mean chemical oxygen demand (COD) removal of 94.8% and the specific CH_4 yield of 0.21 l/g $COD_{removed}$. The CH_4 content in the biogas was increased along the compartments, whereas the percentage of H_2 was decreased, indicating the distribution characteristics of the methanogens occurred longitudinally down the ABR. A high phylogenetic and ecological diversity of methanogens was found in the ABR, and all the detected methanogens were classified into six groups, including Methanomicrobiales, Methanosarcinales, Methanobacteriales, Crenarchaeota, Arc I, and Unidentified. Among the methanogenic population, the acid-tolerant hydrogenotrophic methanogens including *Methanoregula* and *Methanosphaerula* dominated the first two compartments. In the last two compartments, the dominant methanogenic population was *Methanosaeta*, which was the major acetate oxidizer under methanogenic conditions and could promote the formation of granular sludge. The distribution of the hydrogenotrophic (acid-tolerant) and acetotrophic methanogens in sequence along the compartments allowed the ABR to perform more efficiently and steadily.

Key words: Anaerobic baffled reactor, methanogens, diversity, distribution

The production of bioenergy from organic wastes is an essential component in the global development of sustainable energy sources [1]. As an ideal cost-effective biological means to remove organic pollutants in solid waste and wastewater and simultaneously produce methane as an energy source, anaerobic digestion is a prominent bioenergy technology worldwide [10, 18, 33]. The anaerobic conversion of the organic waste to methane requires cooperation of three groups of the microorganisms at least; namely, fermenting bacteria, syntrophic bacteria, and methanogens [37]. The anaerobic respiration of methanogens (methanogenesis) allows them to occupy a physiologically unique niche that is unavailable to Bacteria [23]. They play an important role in the anaerobic food chain, driving anaerobic fermentation by removal of excess H_2, formate, and acetate. However, methanogens are very vulnerable to environmental temperature, pH, and inhibitory chemicals [17, 20, 23, 27]. Therefore, methanogenesis is usually considered as a rate-limiting step for methane fermentation, and high activity of methanogens is beneficial to the performance of an anaerobic digestion process. Moreover, the relative abundance of different types of methanogens in anaerobic digesters can be regulated by the availability of substrates and temperature, pH, hydraulic retention time (HRT), and reactor configuration [13].

The anaerobic baffled reactor is a typical multiphase anaerobic bioreactor consisting of several compartments. The most significant advantage of the ABR is the phase separation, allowing different microbial groups to develop under favorable condition in each compartment [4]. The anaerobic food chain established by the microbe groups located in the compartments allows the ABR to perform more efficiently and steadily, and has attracted increasing interest [4, 18, 35, 36, 41]. The phylogenetic diversity of methanogens in a single-phase anaerobic bioreactor (upflow anaerobic sludge blanket, UASB) has been studied extensively based on cultivation techniques and culture-independent methods [9, 14, 30, 34]. However, there is little information about the phylogenetic diversity of methanogens

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in multiphase anaerobic bioreactors, restricting to some extent the development of this potential technology [41].

In order to get deep understanding about the distribution characteristics of methanogens in multiphase anaerobic bioreactors, an ABR with four compartments was introduced to treat sugar refinery wastewater at mesophilic condition. When the reactor performed steadily at an organic loading rate of 5.33 kg COD/m^3·day, the diversity and distribution of methanogenic archaea were investigated by polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE).

Materials and Methods

Bioreactor System

The ABR used in this research was a lab-scale reactor (620 × 100 × 520 mm, LWH) with a working volume of 27.8 L. It was made of transparent perspex and separated into four equal compartments (named as C1, C2, C3, and C4 in sequence) (Fig. 1). Each compartment was divided by a vertical baffle into a down-flow and an up-flow section with a volume ratio of 1:4. There were four sample outlets equidistant on the wall of each up-flow section. A sedimentation tank with a volume of 1.5 L was attached to C4 for water level control and trapping solids. The feed was pumped to the reactor by a peristaltic pump. The trapped solids were discharged periodically from the sedimentation tank. The reactor was wrapped by electrothermal wire and the temperature in the system was maintained at 35°C ± 1°C by a temperature controller. The evolved biogas in each compartment was collected and led into a waterlock separately. Biogas volume was metered by wet gas meters (Model LML-1; Changchun Filter Co., Ltd.). Waterlocks and wet gas meters were filled with water of pH 3.0, preventing the biogas from dissolution.

Feed Stock

Sugar refinery wastewater was collected from a local beet sugar refinery industry in Harbin, China. Its characteristics had been reported in a previous research [21]. The wastewater was diluted by tap water to a chemical oxygen demand (COD) of 8,000 mg/l, and the COD:N:P in the feed was maintained at 300:5:1 by adding synthetic fertilizer to supply microorganisms with adequate nitrogen and phosphorus. The pH in the influent was adjusted to 7.2 by $NaHCO_3$.

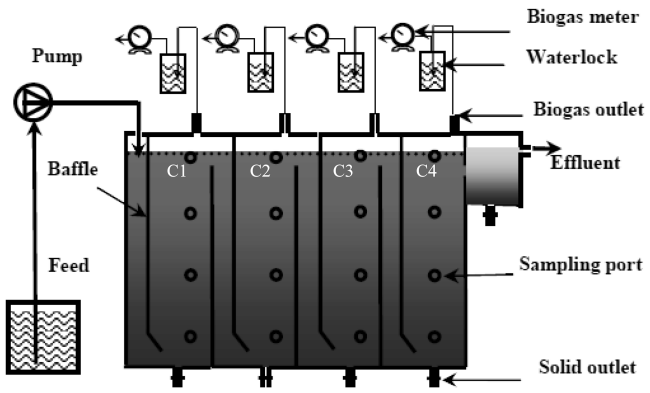

Fig. 1. Schematic diagram of the anaerobic baffled reactor (ABR).

Reactor Operation and Sludge Sampling

After inoculating with the excess sludge obtained from a secondary settling tank of a local brewing wastewater treatment plant in Harbin, China, the ABR was started up at a hydraulic retention time (HRT) of 36 h with an influent COD of 1,000 mg/l. The organic loading rate (OLR) was increased stage by stage to 5.33 kgCOD/m^3·day by enhancing the influent COD to 8,000 mg/l. After two weeks of operation at OLR 5.33 kgCOD/m^3·day, sludge in the ABR was sampled from the bottom sampling port of each compartment for microbial community analysis.

Analytical Methods

COD and pH were measured according to Standard Methods [2]. The biogas composition (CH_4, CO_2, and H_2) were analyzed by a gas chromatograph (Model SC-7; Shandong Lunan Instrument Factory, China) [22]. The soluble intermediate metabolite volatile fatty acids (VFAs) were analyzed by another gas chromatograph (Model GC-112; Shanghai Analytical Apparatus Corporation, China) [22].

DNA Extraction, PCR-DGGE, and Sequencing

Genomic DNA of the sludge samples was extracted using a DNA extraction kit (MO Bio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instruction. The V3 and V5 regions of 16S rRNA genes were amplified by PCR using universal archaeal primers (344F, 5'-ACGGGGYGCAGCAGGCGCGA-3' with a GC clamp and 915R, 5'-GTGCTCCCCCGCCAATTCCT-3') [8]. The PCR amplification was carried out on GeneAmp PCR system 9700 (ABI, USA) in a 50 μl system containing 5 μl of 10× Ex *Taq* buffer, 4 μl of dNTP mixture (2.5 mM), 1 μl of forward primer (20 μM), 1 μl of reverse primer (20 μM), 2.5 ng of DNA template, and 0.15 U of Ex *Taq* DNA polymerase (Takara, Dalian, China). The PCR was denatured for 10 min at 94°C. A total of 30 cycles, each including 1 min at 94°C, 45 s at 55°C, 45 s at 72°C, was followed by a final extension step of 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. Then, the 15 μl samples were loaded and electrophoresed at 120 V and 60°C for 10 h. Gels were silver-stained as described by Bassam *et al*. [5]. The prominent bands were excised and dissolved in 30 μl of 1× TE at 40°C for 3 h, and then centrifuged at 12,000 rpm for 3 min. The 3 μl supernatant was used as the template and PCR amplification was conducted under the same conditions as described above using the same primers. The PCR products were cloned into *E. coli* DH5α after purification by a Gel Extraction Mini kit (Watson Biotechnologies Inc., China) and ligated into the pMD18-T vector (Takara, Dalian, China). Positive clones were detected by ampicillin resistance and blue-white spot screening. Positive clones were sequenced by ABI3730 and gene sequences of the clones were deposited in GenBank under accession numbers JQ799901 to JQ799911. Sequence similarity searches were performed using the BLAST program in GenBank. The phylogenetic tree was constructed by MEGA 3.1 software, referring to the neighbor-joining method.

Table 1. Performance of the ABR during steady operation course.

		Compartment 1	Compartment 2	Compartment 3	Compartment 4
Biogas composition	VFAs in total	1,625 ± 34	2,255 ± 46	886 ± 25	290 ± 10
	Acetate	1,127 ± 33	1,495 ± 34	571 ± 17	134 ± 12
	pH	6.2 ± 0.2	6.0 ± 0.2	7.1 ± 0.1	7.4 ± 0.1
	Biogas production (l/day)	13.9 ± 0.2	18.1 ± 0.1	25.1 ± 0.2	4.2 ± 0.1
	Methane production (l/day)	4.0 ± 0.1	6.7 ± 0.1	13.8 ± 0.1	2.4 ± 0.1
	CH_4 content (%)	28.6 ± 1.2	37.0 ± 1.6	55.0 ± 1.0	56.5 ± 0.5
	CO_2 content (%)	66.2 ± 2.0	60.4 ± 1.4	38.2 ± 0.7	26.8 ± 0.4
	H_2 partial pressure (Pa)	50.0 ± 10	5.0 ± 7	ND	ND
COD	Influent (mg/l)	8,000 ± 210	6,712 ± 116	4,912 ± 97	750 ± 55
	Effluent (mg/l)	6,712 ± 116	4,912 ± 97	750 ± 55	414 ± 30
	Contribution rate to the total removal (%)	16.1 ± 0.6	22.5 ± 0.7	52.0 ± 0.4	4.2 ± 0.5
	Sludge appearance	Flocculent	Flocculent +granule	Granule	Granule

Average value of two weeks during the steady state at an OLR of 5.33 kg COD/m^3·day.
ND, not detected.

RESULTS AND DISCUSSION

Performance of the ABR in Steady State

After attaining steady state for the OLR at 5.33 kg COD/m^3·day, the ABR was operated for another two weeks. Table 1 shows the performance of the bioreactor in the steady state. Higher amounts of VFAs were detected in the first two compartments with a low methane content (28.6%~37.0%), implying an acidogenic phase had been established in them [4, 28]. The yield of VFAs largely led to a low pH in the first two compartments (6.2 and 6.0, separately). Most identified methanogens have been reported to grow and produce methane under neutral or slightly alkaline conditions (pH 6.8–8.5) [10, 13]. However, 4.0 and 6.7 l/day CH_4 were obtained in C1 and C2 at pH 6.0 and 6.2, separately, suggesting that some acid-tolerant methanogens might be located in the first two compartments of the ABR.

The maximum methane yield of 13.8 l/day came up in C3 with a methane content of 55.0%, whereas only 2.4 l/day CH_4 was produced in C4, with a methane content as high as 56.5%, due to scarcity of the substrate (Table 1). Consumption of VFAs led to an increase in pH, with 7.1 in C3 and 7.4 in C4, which were higher than that in the first two compartments. The results suggested that the methanogenic phase had been established in the last two compartments of the ABR [4, 28].

Above all, an observable phase separation of microorganic community occurred in the ABR. The anaerobic food chain established by the acidogenic phase and methanogenic phase allowed the ABR to perform more efficiently and steadily [4, 18, 35, 36, 41] and a mean COD removal of 94.8% was obtained during the steady state at OLR of 5.33 kg COD/m^3·day. The yield and release of biogas, especially CH_4, was the main approach to remove COD from the organic wastewater. As shown in Table 1, the highest contribution rate to the total COD removal of 52.0% was observed in C3, whereas the contribution rate in C1, C2, and C4 was 16.1%, 22.5%, and 4.2%, respectively.

Phylogenetic Diversity of Methanogens in the ABR

Although acidogenesis and methanogenesis dominated the first two compartments and the last two compartments,

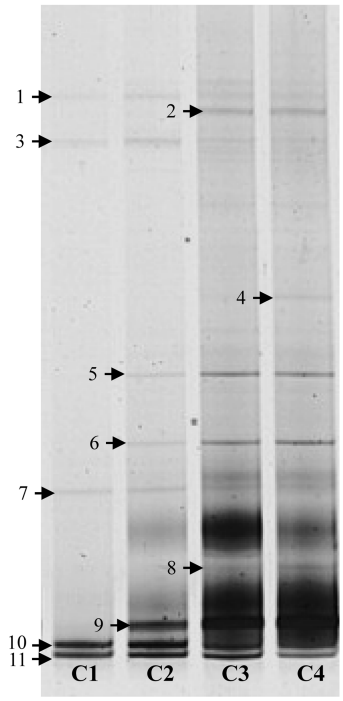

Fig. 2. Archaeal DGGE fingerprinting analysis of sludge samples from each compartment of the ABR.
C1, C2, C3, and C4 represent the four different compartments in the ABR; 1–11 are archaeal bands.

respectively, the methanogenic phenomenon could be observed in each compartment in the ABR. The results suggested that there were some methanogens located in each compartment. To understand the composition and distribution of the methanogens in the bioreactor, sludge sampled from each compartment was analyzed by PCR-DGGE. Eleven methanogenic archaeal bands were collected from the PCR-DGGE gel (Fig. 2) and sequenced. The phylogenetic placement of the 11 sequences and reference 16S rDNA sequences of methanogens in the NCBI database are shown in Fig. 3. Most of the bands belonged to phylum Euryarchaeota and only one band was affiliated with phylum Crenarchaeota.

All of the obtained sequences were grouped into six groups in the phylogenetic tree (Fig. 3). Band 1 had a common node with uncultured Crenarchaeote clone, which was related to the phylum Crenarchaeota. Crenarchaeota archaeal populations were also found to be the dominant archaeal community in an anaerobic reactor for co-digestion of dairy and poultry wastes [41]. Although most of the known methanogens are related to the phylum Euryarchaeota [23–26, 38], Crenarchaeota archaeal populations may play an important role in anaerobic digestion of some organic wastes and further investigation should be carried out in the future. Band 2 exhibited a 99% similarity to uncultured Arc I archaeon within the lineage Arc I. This lineage was also found to dominate in other anaerobic digesters [29]. However, there is little data available about the metabolic capabilities of the Arc I lineage [12, 40]. Band 3 was different from current identified methanogenic groups, forming a unique branch in the phylogenetic tree. It showed a 97% sequence similarity to uncultured archaeon (AB541681) that had previously been found in cattle manure (NCBI database). Band 4 was clustered in the order Methanobacteria, showing 100% similarity with *Methanobacterium beijingense* strain 4-1 isolated from a mesophilic UASB reactor treating beer-manufacture wastewater [24].

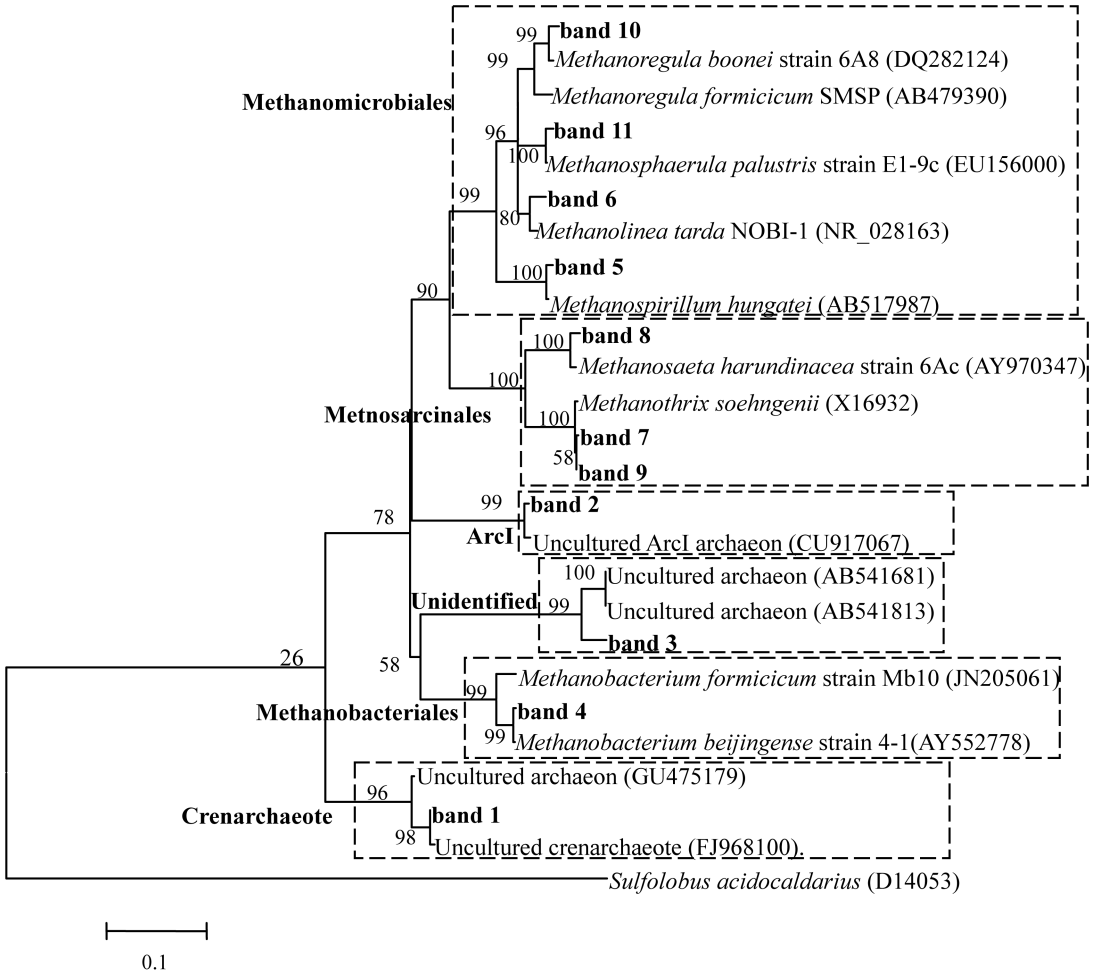

Fig. 3. Phylogenetic tree based on partial 16S rRNA gene sequences.
Bands 1–11 represent different archaeal sequences from the corresponding bands in Fig. 2.

Bands 5, 6, 10, and 11 belonged to the order Methanomicrobiales with a sequence similarity above 98% with *Methanospirillum hungatei*, *Methanolinea tarda* NOBI-1, *Methanoregula boonei* 6A8, and *Methanosphaerula palustris*, seperately. These four methanogens were isolated from different habitats such as puddly soil, methanogenic digester, acidic peat bog, and a minerotrophic fen [6, 7, 15, 38]. Bands 7, 8, and 9 were involved in the order Methanosarcinales, exhibiting a 99% similarity with the methanogens in *Methanosaeta*. Species in this genus have been reported to use acetate as their sole energy source to produce methane [19].

Keyser *et al*. [19] found that the methanogens from three UASB reactors, treating brewery, winery, and peach-lye canning wastewaters, respectively, were related to the orders Methanomicrobiales and Methanosarcinales [19]. The methanogens in two five-compartment fixed-bed anaerobic baffled reactors (FABRs) treating a mixture of molasses and cat food were found belonging to the orders Methanomicrobiales and Methanosarcinales, and phylum Crenarchaeota [39]. Up to now, only 2–4 methanogenic groups were reported involving in single-phase or multiphase anaerobic digesters [3, 19, 28, 39]. It is encouraging that six methanogenic groups were detected in the present ABR treating sugar refinery wastewater, indicating a high phylogenetic diversity of the methanogens in the ABR.

Distribution of Methanogens in the ABR

As shown in Table 2 and Fig. 2, uncultured Crenarchaeote (band 1), *Methanothrix soehngenii* (band 7), and *Methanosphaerula palustris* (band 11) existed in all the compartments, indicating that they have a wide niche. Among them, *Methanothrix soehngenii* is an aceticlastic methanogen, and *Methanosphaerula palustris* is a slightly acidiphilic hydrogenotrophic methanogen with a pH range of 4.8~6.4 for growth [7]. The ecological features of the species in phylum Crenarchaeota need to be explored in the future.

Methanoregula boonei (band 10) was another major methanogenic community in the first two compartments with pH 6.0~6.2 (Fig. 2, Table 1). It used formate and H_2/CO_2 as substrate with the optimum pH 5.5 for methane production [38]. Band 3, which was related to an unidentified group, is also existed in C1 and C2, indicating the band 3 represented a novel acid-tolerant methanogenic group. It was confirmed that some acid-tolerant methanogens were located in the ABR, resulting in an observable methane yield in the first two compartments (Table 1).

In C3 and C4, *Methanospirillum hungatei* (band 5) and *Methanolinea tarda* (band 6) were also the dominant hydrogenotrophic methanogens, besides *Methanosphaerula palustris* (band 11) (Table 2 and Fig. 2). Both *Methanospirillum hungatei* and *Methanolinea tarda* utilize H_2/CO_2 and formate for growth and methane production with optimum pH 7.5 and pH range of 6.7–8.0, respectively [15, 16]. The lineage Arc I (band 2) could also be observed in the last two compartments. This group had been reported to grow on formate or H_2/CO_2 [11], and the ability to grow on acetate is still unconfirmed to now [29]. It was noticeable that the *Methanobacterium beijingense* (band 4), which used H_2/CO_2 and formate for growth and produced methane with optimum pH 7.5–7.7, was unique for C4 [24].

In addition, it can be seen from Table 2 and Fig. 2 that *Methanosaeta* (band 9) was the predominant acetotrophic methanogens in the last two compartments. *Methanosaeta* can use acetate at concentration as low as 5–20 μM [23]. It was reported that the optimum pH for acetotrophic methanogens was between 6.6 and 7.4, and their methanogenesis would be inhibited badly when the pH was below 6.2 [13]. A previous quantitative analysis demonstrated that *Methanosaeta* spp. was the dominant aceticlastic methanogen in anaerobic bioreactors with low acetate concentrations and the *Methanosaeta* spp. levels were higher in bioreactors with granular sludge than that with flocculent sludge [13, 42]. This was consistent with the present study. Granular sludge was formed in the last two compartments

Table 2. Sequences of excised DGGE bands.

Band	GenBank Accession No.	Similar sequence (Accession No.)	Similarity (%)	Source
1	JQ799911	Uncultured Crenarchaeote (FJ968100)	99	C1/C2/C3/C4
2	JQ799910	Uncultured ArcI archaeon (CU917067)	99	C3/C4
3	JQ799901	Uncultured archaeon (AB541681)	97	C1/C2
4	JQ799902	*Methanobacterium beijingense* strain 4-1 (AY552778)	100	C4
5	JQ799904	*Methanospirillum hungatei* (AB517987)	99	C2/C3/C4
6	JQ799906	*Methanolinea tarda* NOBI-1 (NR_028163)	98	C2/C3/C4
7	JQ799909	*Methanothrix soehngenii* (X16932)	99	C1/C2/C3/C4
8	JQ799908	*Methanosaeta harundinacea* strain 6Ac (AY970347)	99	C3/C4
9	JQ799907	*Methanothrix soehngenii* (X16932)	99	C2/C3/C4
10	JQ799903	*Methanoregula boonei* strain 6A8 (DQ282124)	99	C1/C2/C3
11	JQ799905	*Methanosphaerula palustris* (EU156000)	99	C1/C2/C3/C4

when the acetate concentration was less than 571 mg/l (Table 1). Granulation allowed the ABR to retain a higher diversity and create spatial juxtapositioning of various populations.

Most methanogens grow optimally under neutral to slightly alkaline conditions and are very vulnerable to pH. Their growth rate will be seriously reduced below pH 6.6 [17, 20, 23, 27]. Thus, acid-tolerant methanogens are desired to improve the efficiency and stability of anaerobic digesters [32]. In the present ABR, some acid-tolerant methanogens were enriched in the first two compartments with a low pH (6.0–6.2). These acid-tolerant methanogens allowed the first two compartments a contribution rate of 38.6% to the total COD removal in the ABR (Table 1). Compartmentalization in the ABR can create habitats for methanogens with various niches. Therefore, some acid-tolerant methanogens were enriched in the acidogenic phase, due to the unique construction of ABR. However, only a few pure cultures of acid-tolerant methanogens have been obtained up to now, limiting the understanding of their ecophysiological characteristics [25, 31].

Above all, the unique compartmentalization in construction allowed methanogens with various ecophysiological characteristics to distribute in their corresponding compartment, resulting in a high phylogenetic diversity of methanogens in the ABR treating sugar refinery wastewater. Six groups of methanogens were found in the ABR by PCR-DGGE. Methanomicrobiales and Methanosarcinales-related population containing five genera were the dominant methanogens in the ABR. Some acid-tolerant methanogens were enriched in the first two compartments with pH 6.0–6.2, devoting to a CH_4 yield of 10.7 l/day. The hydrogenotrophic methanogens were the dominant methanogenic community in the first two compartments, whereas acetoclastic methanogens were the predominant population in the last two compartments. Methanogens with different ecophysiological features were distributed longitudinally down the ABR, laying a foundation for the efficient and steady operation of the ABR.

Acknowledgments

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