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RESEARCH ARTICLE

Microbial community analysis in a combined anaerobic and aerobic digestion system for treatment of cellulosic ethanol production wastewater

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Abstract This study investigated the microbial diversity established in a combined system composed of a continuous stirred tank reactor (CSTR), expanded granular sludge bed (EGSB) reactor, and sequencing batch reactor (SBR) for treatment of cellulosic ethanol production wastewater. Excellent wastewater treatment performance was obtained in the combined system, which showed a high chemical oxygen demand removal efficiency of 95.8 % and completely eliminated most complex organics revealed by gas chromatography–mass spectrometry (GC–MS). Denaturing gradient gel electrophoresis (DGGE) analysis revealed differences in the microbial community structures of the three reactors. Further identification of the microbial populations suggested that the presence of *Lactobacillus* and *Prevotella* in CSTR played an active role in the production of volatile fatty acids (VFAs). The most diverse microorganisms with analogous distribution patterns of different layers were observed in the EGSB reactor, and bacteria affiliated with *Firmicutes*, *Synergistetes*, and *Thermotogae* were associated with production of acetate and carbon dioxide/hydrogen, while all acetoclastic methanogens identified belonged to *Methanosaetaceae*. Overall, microorganisms associated with the ability to degrade cellulose, hemicellulose, and other biomass-derived organic carbons were observed in the combined system. The results presented herein will facilitate the development of an improved cellulosic ethanol production wastewater treatment system.

Keywords Continuous stirred tank reactor · Expanded granular sludge bed · Sequencing batch reactor · Cellulosic ethanol · Wastewater treatment · Microbial community

Introduction

Cellulosic ethanol is considered a promising source of substitute energy because ethanol is the only form of biomass-based liquid fuel that can be used for vehicular power, and there is an abundance of lignocellulosic resources (Dwivedi et al. 2009; Mabee et al. 2011). However, substantial increases in cellulosic ethanol production will also result in large quantities of high-strength wastewater. More than 20 L of wastewater is generated for each liter of ethanol produced. Based on the target of producing 21 billion gallons of biofuels from cellulosic feedstocks by 2022 in the USA, more than 420 billion gallons of wastewater will be produced from the cellulosic ethanol production process, resulting in the need for highly efficient treatment techniques (Administration 2011; Dwivedi et al. 2009; Wilkie et al. 2000).

Cellulosic ethanol production wastewater is difficult to treat because it has a low pH, high color, and high chemical oxygen demand (COD), which is usually combined with dissolved organic and inorganic materials (Kharayat 2012; Vanhaandel and Catunda 1994). In addition, cellulosic materials usually require pretreatment before saccharification and ethanol fermentation. The by-products, such as phenolic compounds, metal ions, and aldehydes, will be liberated during the pretreatment process (Wilkie et al. 2000). These by-products

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also exist in cellulosic ethanol production wastewater and have inhibitory effects on microbial activity, resulting in difficult wastewater treatment.

Investigations of the treatment of cellulosic ethanol production wastewater are extremely limited. Most studies conducted to date have been associated with the adaptation of anaerobic digestion (AD) for cellulosic ethanol production wastewater treatment, with COD removal efficiencies ranging from 82 to 92 % (Barta et al. 2010; Tian et al. 2013; Wilkie et al. 2000). Obviously, the COD removal efficiencies of AD in terms of the conversion of complex organic or inorganic materials contained in wastewater are highly reliant on the microbial community. Nevertheless, information describing the microbial communities present in AD for cellulosic ethanol production wastewater treatment is extremely limited. In this study, a combined system composed of a continuous stirred tank reactor (CSTR), expanded granular sludge bed (EGSB) reactor, and sequencing batch reactor (SBR) was employed to treat cellulosic ethanol production wastewater. To better understand the functional microbial populations in reactors, gas chromatography–mass spectrometry (GC–MS) and denaturing gradient gel electrophoresis (DGGE) analysis were applied to study the microbial community structure and corresponding function in the three reactors. The results of the present study will facilitate development of a well-operated system for cellulosic ethanol production wastewater treatment.

Material and methods

Wastewater and treatment technology

The wastewater for this study was collected from a cellulosic ethanol plant (latitude 45° 10′–46° 20′ N and longitude 125° 22′–126° 22′ E) located in Heilongjiang province, China, where corn stover is used as a raw material for ethanol manufacturing. The cellulosic ethanol production wastewater was characterized as follows: chemical oxygen demand (COD) 165,806 mg/L, biochemical oxygen demand (BOD) 44,280 mg/L, pH 3.97, total nitrogen 717.7 mg/L, total phosphorus 120 mg/L.

A combined system of CSTR–EGSB–SBR, designed as schematically shown in Fig. 1, was applied for cellulosic ethanol wastewater treatment: CSTR–EGSB for anaerobic pretreatment (the CSTR for acidogenesis of the organic matter, while the EGSB for methanogenesis) and SBR for aerobic post-treatment. Anaerobic reactors were operated under mesophilic condition (35±1 °C), while aerobic reactor was operated at ambient temperature (25±1 °C). The effective volumes of the CSTR, EGSB, and SBR were 2, 4, and 15 L, respectively. The CSTR was seeded to 50 % (*v*/*v*) of its working volume with granular sludge (10.6 g VSS/L) obtained from the internal circulation (IC) reactor treating fermentation wastewater (latitude 45° 10′–46° 20′ N and longitude 125° 22′–126° 22′ E). The EGSB was seeded to 70 % (*v*/*v*) of its working volume with granular sludge (18.3 g VSS/L) obtained from the same IC reactor. Subsequent SBR was initially inoculated with activated sludge (6.37 g VSS/L) obtained from the municipal sewage treatment plant (latitude 45° 49′ N and longitude 126° 42′ E). For the system operation, during the initial stage of approximately 60 days, mixed wastewater (diluted wastewater and sucrose artificial wastewater) was introduced to the CSTR for microbial acclimation and organic loading rate (OLR) increase (4.35 kg COD/(m^3 day)–41.76 kg COD/(m^3 day)). Once the system performance was stabilized with consistent removal of COD, diluted original wastewater without mixing was fed to the CSTR, and the organic loading rate (OLR) of steady state (42 days) is 40 kg COD/(m^3 day).

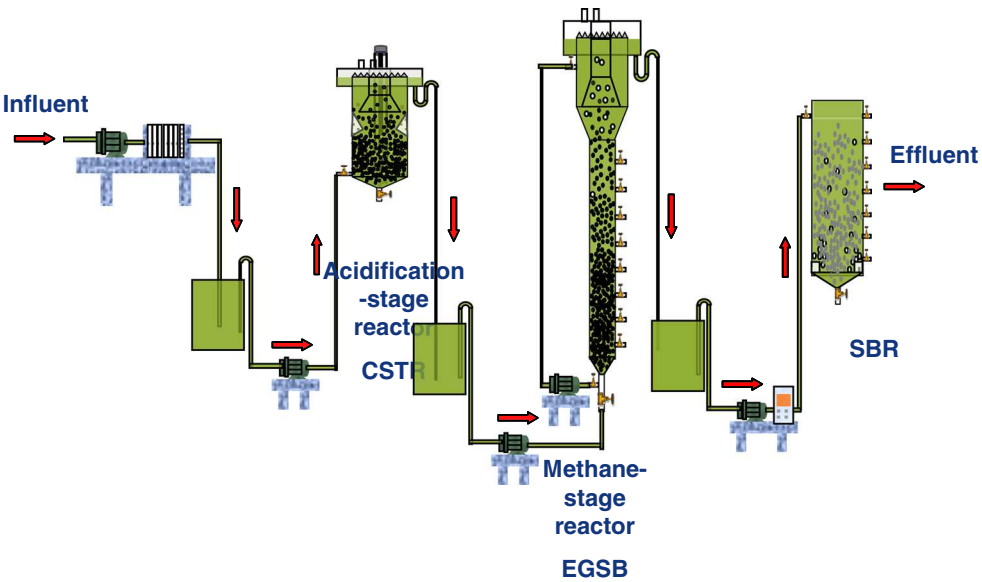

Fig. 1 Schematic diagram of the CSTR–EGSB–SBR treating cellulosic ethanol manufacturing wastewater

For the EGSB reactor, the OLR was step-wisely increased and stabled at 17 kg COD/(m^3 day). The combined CSTR–EGSB–SBR system was operated for 139 days at hydraulic retention time (HRT) of 11.5, 24, and 12 h (aeration 8 h, settling 4 h) respectively. The influent of combined system was diluted with domestic wastewater (Table 3S) to a COD of 12,000 mg/L based on the OLR of the CSTR, and the pH was adjusted to 6–6.5 by the addition of $NaHCO_3$.

DNA extraction and PCR-DGGE analysis

Sludge samples were taken from the three reactors at the end of steady state for microbial community analysis. The sludge was collected from four sampling points in the EGSB reactor and named from top to bottom as levels A–D. Genomic DNA of the sludge samples was extracted according to the procedures as previously reported (Lu et al. 2012). The concentration and purity of DNA were measured by NanoDrop® Microspectrophotometry ND-2000 (Thermo Fisher Scientific, USA).

Microbial communities were examined by DGGE analysis of PCR amplified 16S rRNA gene fragments from each reactor as previously described (Zhu et al. 2014). The 16S rRNA genes for DGGE were amplified using primers 341F with a GC clamp (5′-CCTACGGGAGGCAGCAG-3′) and 907R (5′-CCGTCAATTCMTTTGAGTTT-3′) for bacteria, 344F with a GC clamp (5′-ACGGGGYGCAGCAGGCGCGA-3′), and 915R (5′-GTGCTCCCCCGCCAATTCCT-3′) for archaea. Approximately 500 ng of PCR products was separated as follows: 8 % (*w/v*) polyacrylamide gels and denaturing gradient from 40 to 60 % were used; gels were electrophoresed in 1× TAE buffer at 60 °C and 90 V for 12 h using Bio-Rad DCode Universal Detection Mutation system (Bio-Rad, Hercules, CA, USA), after which the gels were stained with silver and photographed.

DGGE gel images were analyzed with Quantity One software version 4.6.2 (Bio-Rad, USA) (Wang et al. 2015). The software was used to detect bands in each lane using a match tolerance of 2 %. A similarity matrix was constructed using Dice's similarity coefficient. This was defined as $S_{i,j}=2n_{i,j}/(2n_{i,j}+n_i+n_j)$ between bands of the lanes i and j, where n_i was the number of bands found only in lane i, n_j was the number of bands found only in lane j, and $n_{i,j}$ was the number of bands shared between the two lanes (Roeder et al. 2010). For clustering the unweighted pair group method with arithmetic mean (UPGMA), method was applied (Wang et al. 2015). The Shannon index and Simpson index were estimated using $H'=-\sum p_i \ln p_i$ and $D=\sum p_i^2$, respectively (Hill et al. 2003). The indices were calculated based on band intensities of the DGGE profiles (Ivone and Conceição 2013), which measured with the aid of Quantity One (Wang et al. 2015).

For prominent band analysis in DGGE, all non-overlapping bands from DGGE profiles were cut off and sequenced as described by Zhu et al. (2014). The sequences were aligned and compared in the GenBank database using BLAST programs of the National Center for Biotechnology Information (NCBI). In order to compare the 16S rRNA gene sequences of bands from different samples, nucleotide sequences were aligned using ClustalW from MEGA software, version 5.20 (Tamura et al. 2011). Dendrogram representations were obtained after pairwise and multiple sequence alignment on the basis of the model of Jukes and Cantor (1969) and neighbor-joining method (Jukes and Cantor 1969). The methods maximum parsimony and maximum likelihood were also used to assess the tree stability. The type strains of the closest neighbors of each band were added to the dendrogram in order to support the identification of the bands under study (Falcone-Dias et al. 2012). Non-homologous and ambiguous nucleotide positions were excluded from the calculations, and bootstrap values, generated from 1000 re-samplings, at or above 50 % were indicated at the branch points. All sequences included in this paper were deposited to Genbank under accession numbers KP101324 to KP101362.

Analytical methods

The pH value was measured using the digital pH meter (PHS-3C, China). COD of influent and effluent was determined according to the procedures described in Standard Methods (APHA 1995). The total COD removal efficiency was

Table 1 Performance of the combination system during steady operation course

Variables	Influent	CSTR	EGSB	SBR
Effluent COD (mg/ L)	11610 ± 703	8324 ± 575	1163 ± 166	493 ± 72
COD removal rate (%)		28.2 ± 4.6	90.0 ± 1.3	95.8 ± 0.5
Biogas production (L/day)		–[a]	28.7 ± 2.5	–[a]
pH	6.3 ± 0.1	5.0 ± 0.1	7.8 ± 0.2,	8.1 ± 0.1
VFAs in total (mg/ L)	781 ± 58	4506 ± 294	332 ± 18	–[a]
Acetate (mg/ L)	472 ± 12	3364 ± 305	182 ± 10	
Oxidation reduction potential (ORP) (mV)		−299 ± 15	Up (−503 ± 11), down (−456 ± 14)	

Average value of steady state (42 days)

[a] Not detected

calculated based on influent and effluent of the system, while contribution rate of reactor was calculated based on influent and effluent of each individual reactor. The biogas composition (CH_4 and CO_2) and the soluble intermediate metabolite volatile fatty acids (VFAs) were analyzed by a gas chromatograph (GC7890A, Agilent Technologies, USA) with thermal conductivity detector (TCD) and flame ionization detector (FID), respectively. An Agilent 6890GC/5973MSD (Agilent, USA) was employed to identify the majority of organic compounds. The morphological characteristics of microorganisms were observed via scanning electron microscopy (SEM) (S-3400N, Hitachi High-Technologies Corporation, Japan).

All statistical analyses were done using SPSS software by one-way analysis of variance (ANOVA) to determine the significant performance of reactors on measured parameters at the 1 % level.

Results and discussion

Performance of the combined system

The combined CSTR–EGSB–SBR system was operated for 139 days, and the main operational characteristics of steady state (42 days) are summarized in Table 1. A total COD removal efficiency of 95.8 % (Fig. 2) was obtained, and contribution of the CSTR and the EGSB reactor was 28.2 and 86.1 %, respectively. The CSTR was supposed to obtain high hydrolytic and fermenting stability, while the EGSB reactor revealed a positive contribution to COD removal. It is likely that two phase system (CSTR–EGSB) achieved the physical separation of acid-forming and the methane-forming microorganisms (Demirel and Yenigün 2002). To further increase COD removal from the cellulosic ethanol wastewater, a SBR, as aerobic post-treatment, was operated, and the contribution rate of the COD removal was 57.1 %. The sequential anaerobic–aerobic processes investigated in the present study

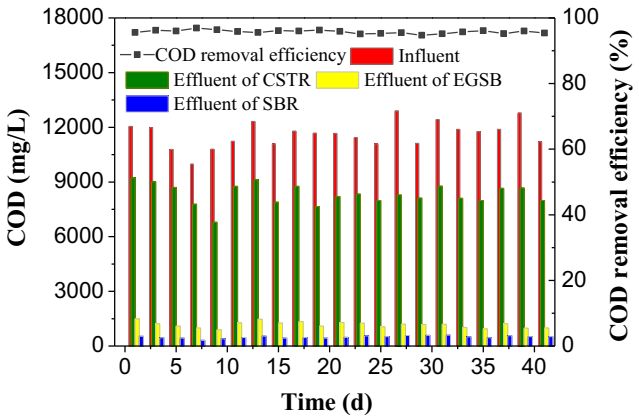

Fig. 2 Variation of the COD of steady state (42 days)in influent and effluent of combined system

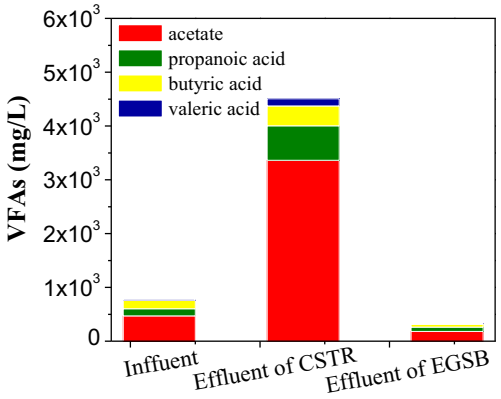

Fig. 3 Variation of the volatile fatty acids concentration in influent and effluent of combined system

achieved higher treatment efficiencies (95.8 % total COD removal) in treating ethanol wastewater from cellulosic feedstocks when compared with the conventional anaerobic applications with COD removal rates ranging from 82 to 92 % (Barta et al. 2010; Tian et al. 2013; Wilkie et al. 2000).

The COD removal efficiency in the CSTR was not expected to be high since the amount of solubilized organic material was converted to volatile fatty acids (VFAs). A considerable amount of VFAs was detected in the CSTR (4506.5 mg/L), indicating that they comprised the majority of soluble products. Acetate was the dominant constituent of the VFAs, comprising an average of 74.6 %, which is significantly (p 0.01) higher than the proportion of propionic acid, butyric acid, and valeric acid (made up the remaining 25.4 %) (Fig. 3). High proportion of acetate in CSTR might facilitate the activity of acetate-utilizing methanogenic archaea observed in the EGSB reactor (Ahring et al. 2001).

The VFAs formation during acidogenesis of the organic matter was actually the precursor to methanogenesis. Significant decreases in VFAs concentration were observed in the EGSB reactor, while increases in pH and biogas production were observed. The rate of biogas production was 28.7 L/day, and the typical composition of the biogas was 69 % methane,

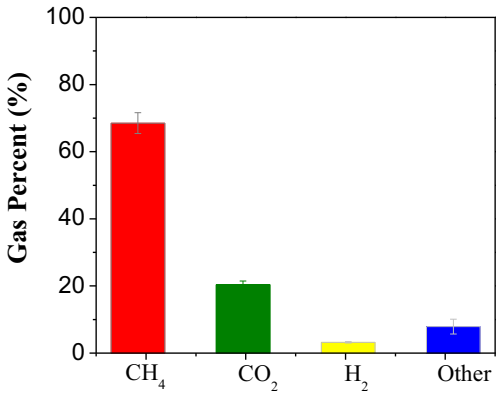

Fig. 4 The typical composition of the biogas of the EGSB reactor at the steady state operation

20 % carbon dioxide, 3 % hydrogen, and 8 % other gas (Fig. 4). Biogas production with concurrent stable organic decomposition was observed in the EGSB reactor, and a COD removal efficiency of greater than 90% was ultimately maintained in the two-stage CSTR–EGSB reactors. The aerobic post-treatment of anaerobically pre-treated cellulose ethanol wastewater resulted in a COD removal efficiency of about 57 %.

GC–MS analysis of the raw wastewater and biotreatment effluent

To gain more insight into which compounds are eliminated in the two-stage anaerobic and aerobic digestion, GC–MS analysis was carried out. Table 2 lists the majority of compounds of raw wastewater and effluent of the combined system and their corresponding peak areas with GC–MS analysis. The compounds were grouped according to their principal functional groups.

Carboxylic acids and alcohols were the main compounds present in raw wastewater, comprising 20.4 and 71.3 %, respectively. The presence of organic acids explained the low pH well. Following CSTR treatment, most complex organics were converted into monomers, usually in terms of VFAs, by the diverse microbial communities, indicating the effective acidogenesis of the CSTR. In addition, there was a large quantity of phenolic compounds in raw wastewater, which were converted from lignocellulosic materials degradation. Phenolic compounds are known to inhibit biological activity (Wilkie

Table 2 Organic matter analysis of raw and effluent of the combined system

Compounds	Raw (peak area)	Effluent (peak area)		
		CSTR	EGSB	SBR
Carboxylic acids				
Lactic acid	35,621,915	–	–	–
2-Hydroxy-2-methylbutyric acid	9,672,694	–	–	–
2-Hydroxy-4-methylpentanoic acid	11,671,975	–	–	–
2-Hydroxyhexanoic acid	3,701,933	–	–	–
Benzoic acid	9,257,645	–	–	–
D-(+)-Phenyllactic acid	20,722,847	–	–	–
Azelaic acid	13,262,951	–	–	–
Palmitic acid	4,503,758	–	–	–
2-Furoic acid	16,971,437	–	–	–
3-(4-Hydroxyphenyl)propionic acid	80,401,205	–	–	–
(4-Hydroxy-3-methoxyphenyl)propionic acid	337,174,324	–	–	–
Hexanoic acid	–	–	5,563,417	–
Octanoic acid	–	–	4,450,490	–
Alcohols				
Isobutyl alcohol	41,229,046	–	–	–
1-Pentanol	11,542,350	–	–	–
Isoamyl alcohol	40,512,239	–	–	–
2-Pentanol	1,391,907,000	–	–	–
Propylene glycol	75,218,695	–	–	–
2-Hexanol	26,006,812	–	–	–
2,3-Butanediol	251,898,498	–	–	–
2-Methyl-1,3-butanediol	5,707,468	–	–	–
Phenethyl alcohol	23,009,747	–	–	–
Glycerol	3,857,698	–	–	–
1,2,5-Pentanetriol	17,169,042	–	–	–
Furfuryl alcohol	–	3,441,276	–	–
3-(3-Methoxy-4-hydroxyphenyl)-1-propanol	7,923,786	–	–	–
Acetic acid 3-hydroxybutyl ester	2,905,213	–	–	–
3,5-Dimethylphenol	203,100,214	22,903,011	–	–
4-Ethylphenol	–	428,452	–	–
1-(4-Hydroxyphenyl)ethanone	2,845,833	–	–	–
a-D-Arabinopyranose	10,767,738	–	–	–

et al. 2000). Most phenolic compounds were degraded in the EGSB, while the majority of complex organics were completely eliminated after treatment by the combined system, indicating effective degradation and performance of the two-stage anaerobic and aerobic digestion.

Analysis of microbial community

The removal of organic compounds by a combined anaerobic and aerobic process was closely related to diverse microbial communities responsible for hydrolysis, acidification, and gasification of VFAs (Fox and Pohland 1994). DGGE analysis of the 16S rRNA gene was used to investigate differences in the microbial communities among three reactors (Fig. 5). The resultant dendrogram showed that the DGGE bands of the bacteria were clustered into two groups, and that this clustering was mainly dependent on the reactors. Moreover, the bacterial similarity between the CSTR and EGSB reactor, with the same inocula, was 0.00 (Fig. 5a). It is likely that the two-phase system achieves the physical separation of acid-forming and the methane-forming microorganisms in two separate reactors (Demirel and Yenigün 2002). In addition, the archaea were clustered into only one group due to the high similarity with different layers (0.81) (Fig. 5b). It is likely that the archaeal diversity in the EGSB was limited compared to bacteria. This finding is in agreement with the results of previous study (Leclerc et al. 2004). Analysis of the DGGE patterns using the Shannon index (*H′*) and Simpson's index (*D*) revealed that the bacterial communities of the CSTR had lower diversity (1.30 and 0.35, respectively) than other two reactors (Fig. 5a). In the EGSB

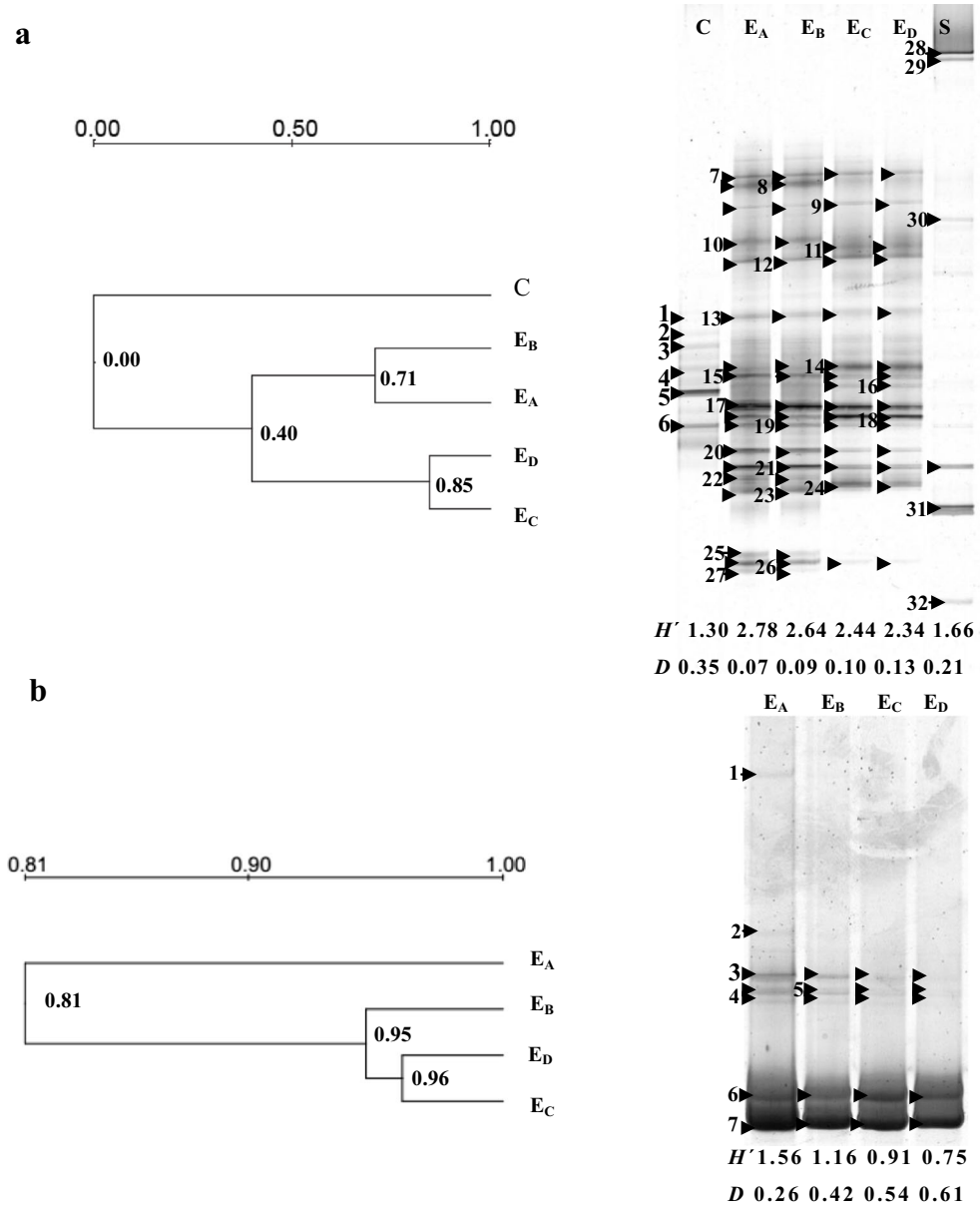

Fig. 5 Microbial community analysis by PCR-DGGE and clustering. Some prominent DGGE bands indicated by *arrows* and *numbers* were identified (Tables 1S and 2S). **a** Bacterial community analysis. **b** Archaeal community analysis. **c** Sample from CSTR reactor. (*EA-D*) Samples from top to bottom of EGSB reactor. (*S*) Sample from SBR reactor. (*H′*) Shannon index and (*D*) Simpson's index of microbial diversity

reactor, the diversity of bacteria and archaea showed analogous change patterns gradually increasing from the bottom to the top. However, bacterial diversity was generally higher than that of archaeal communities of EGSB reactor. Moreover, the lowest diversity (H' 2.34; D 0.13) of bacteria in EGSB reactor was even higher than that of archaea (H' 0.75; D 0.61) (Fig. 5). The Shannon index (H') and Simpson's index (D) of SBR were 1.66 and 0.21, respectively. To obtain further insight into the taxa prevailing in each sample, 32 distinct and non-overlapping bands out of the 78 PCR-DGGE band classes detected were excised, cloned, and sequenced for bacteria, while 7 of the 22 PCR-DGGE bands were excised and sequenced for archaea. All sequences could be classified into eight bacterial and two archaeal phyla. Sequences affiliated with these phyla were reconstructed into phylogenetic trees (Figs. 6 and 7).

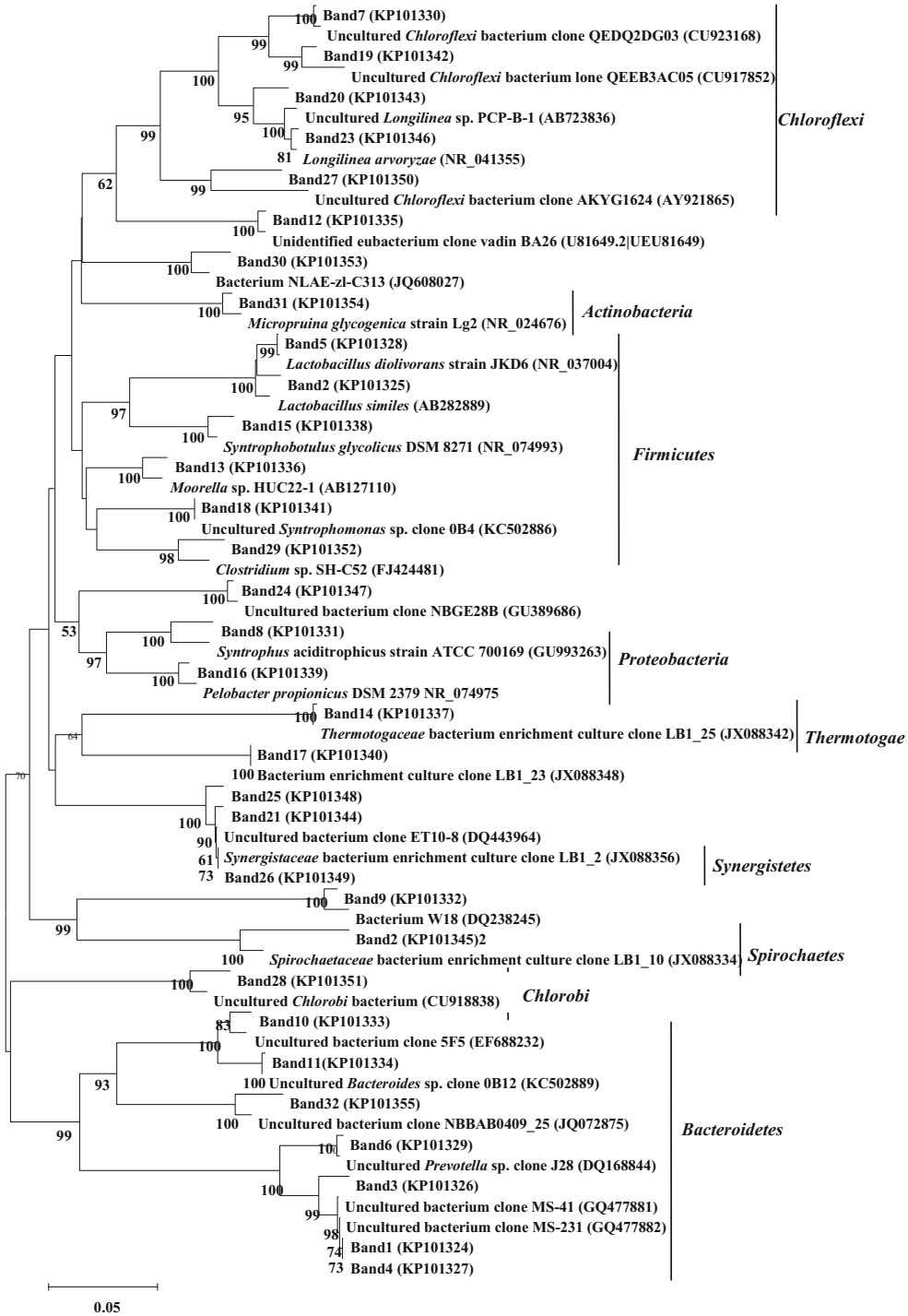

Fig. 6 Phylogenetic tree based on partial 16S rRNA gene sequences. Bands 1–32 represent different bacteria sequences from the corresponding bands in Fig. 5a

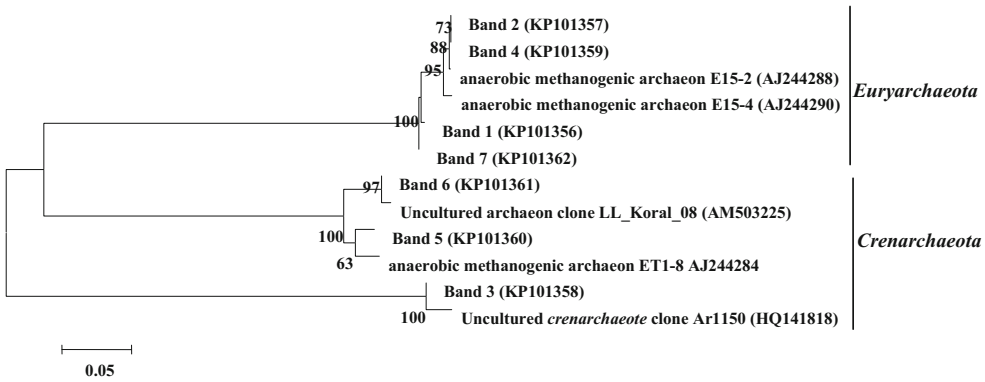

Fig. 7 Phylogenetic tree based on partial 16S rRNA gene sequences. Bands 1–7 represent different archaeal sequences from the corresponding bands in Fig. 5b

Phylogenetic diversity of bacteria in CSTR

Complex organic materials converted into VFAs were associated with microorganisms of the CSTR. The *Firmicutes* and *Bacteroidetes* communities were only observed in the CSTR (Figs. 5 and 6, Table 1S), which indicated a lower diversity of microorganisms. *Lactobacillus* sp. (bands 2 and 5) and *Prevotella* (band 6) belonging to the phylum *Firmicutes* and *Bacteroidetes,* respectively, were identified. On the other hand, normally rod-shaped *Lactobacillus* and *Prevotella* were observed upon SEM (Fig. 1S), which agree with those of previous studies regarding the morphology of these microorganisms (Bottazzi 1988; Moore et al. 1994).

The presence of *Prevotella* (band 6) in CSTR was consistent with the previous finding that the phylum *Bacteroidetes* was the major active taxon present during anoxic cellulose treatment (Schellenberger et al. 2010). *Prevotella* has been described as a hemicelluloses decomposing bacterium (Ueki et al. 2007). The main function of phylogenetic groups of *Prevotella* is the production of short-chain fatty acids, and the main fermentation products are acetate and succinic acid (Eusebio et al. 2011; Shen et al. 2013), which was likely associated with the high proportion of acetate production in the CSTR (Fig. 3).

Phylogenetic diversity of microorganisms in EGSB reactor

The high contribution to COD removal observed in the EGSB reactor might be associated with the great diversity of microorganisms. Samples from EGSB produced a complex fingerprint composed of a large number of bands. Subsequent sequence analysis of these bands revealed that primary bacteria were affiliated with *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Bacteroidetes*, *Synergistetes*, *Thermotogae*, and *Spirochaetes*. In addition, samples from different sampling points generated similar locations of a large number of bands and analogous band patterns.

As shown in Fig. 5a, the relative intensity of most of the bands was gradually increased from bottom to top. Species (bands 7, 19, and 20) belonging to class *Anaerolineae* (phyla *Chloroflexi*) were mainly located in the upper layer, which was similar to the distribution pattern of microbial diversity observed at the phylum level in a full-scale UASB reactor for treatment of domestic wastewater (de Lucena et al. 2011). This was likely due to the strictly anaerobic environment in the upper layer of the EGSB (Gregoire et al. 2011) with the lower ORP value (−503 mV) (Table 1). Furthermore, species (bands 13 and 15) belonging to *Firmicutes* were associated with biogas production of hydrogen and carbon dioxide in the EGSB reactor, ending in reduction of acetate (Table 2). These findings agree with those of previous studies regarding the role of these microorganisms, which are known to be effective for degradation of complex organic materials and acetate to hydrogen and carbon dioxide (Jang et al. 2014; Narihiro et al. 2012). In addition, *Moorella thermoacetica* (band 13) is responsible for fermented acetate production. Specifically, this strain can directly convert cellulosic biomass to acetate (Karita et al. 2003) and may exhibit excellent degradation of cellulosic materials in cellulosic ethanol production wastewater.

Conversely, species (bands 14 and 18) belonging to phyla *Thermotogae* and *Firmicutes* were mainly located in the bottom layer, and the relative intensity was first found to be gradually decreasing from bottom to top. It is possible that this result was due to preferential ecological niche (the high content of substrate in the bottom layer), just as the archaeal species were able to occupy the ecological niche of methanogenesis in digesters (Leclerc et al. 2004). The concentration of butyric acid in influent and effluent of the EGSB reactor was 369.4 and 50.4 mg/L, respectively. Species affiliated with *Syntrophomonas* (band 18) and *Thermotogaceae* (band 14) had genetic potential to oxidize butyrate as the substrate to acetate and carbon dioxide/hydrogen (Lykidis et al. 2011; Zou et al. 2003). This may explain the high reduction (86.4 %) of butyric acid (Table 1) in the EGSB effluent.

Similar to the distribution of bacteria in the EGSB, the diversity and relative intensity of corresponding bands of archaea gradually increased from bottom to top (Fig. 5b). This was also attributed to the strictly anaerobic environment in the upper layer with lower ORP value (−503 mV) (Table 1). The

archaeal phyla *Euryarchaeota* and *Crenarchaeota* were observed in the EGSB reactor, and all archaeal sequences affiliated with these phyla were reconstructed into phylogenetic trees (Fig. 7).

Species (bands 1, 2, 4, and 7) of the phylum *Euryarchaeota* were found to be associated with acetoclastic methanogenesis, and all acetoclastic methanogens identified belonged to *Methanosaetaceae*. These findings agreed with those of previous studies for acetoclastic methanogens, which were observed in typical granules of the stabile methanogenic reactor (two-stage anaerobic digestion) (Demirel and Scherer 2008; Zheng and Raskin 2000). *Methanosaeta* species dominated in low concentrations of acetate, and their numbers decreased fast as the acetate concentration increased (Demirel and Scherer 2008). This may explain the presence of *Methanosaeta* spp. in EGSB reactor, where acetate concentration was gradually depleted, and that of effluent was 182 mg/L (Table 1). Furthermore, the pH of the anaerobic digestion affects the presence and the activity of the acetotrophic methanogens, and the optimum pH for *Methanosaeta* spp. was above 7.0, while that for *Methanosarcina* spp. was below 7.0 (Demirel and Scherer 2008). The effluent of the EGSB reactor in this study was 7.8 (Table 1), which may further interpret the presence of the *Methanosaetaceae*. In addition, sequences categorized in *Euryarchaeota* and *Crenarchaeota* species showed similarities of at least 97 % with anaerobic methanogenic archaea isolated from stable cellulose-degrading enrichment cultures (Chin et al. 1999). It is likely that methanogenic archaea in the EGSB would be associated with the capability to degrade cellulose, hemicellulose, and other biomass-derived organic carbons, and that they were the dominant members of the archaeal community present during the anaerobic process.

Phylogenetic diversity of bacteria in SBR reactor

Different from anaerobic digestion, the COD removal efficiency in the SBR was not expected to be high (57.1 %) that might be associated with less diversity among microorganisms (Calli et al. 2003; Pholchan et al. 2010) (Fig. 5a). *Chlorobi*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* communities were observed in the SBR. *Clostridium* (band 29) and *Micropruina glycogenica* (band 31) belonging to the phyla *Firmicutes* and *Actinobacteria*, respectively, were reported as aerobic cellulolytic and lignocellulolytic bacteria and played a leading role in aerobic lignocellulolytic wastewater degradation (Chin et al. 1999). In addition, recalcitrant dissolved organic materials generated in response to repolymerization of compounds were present in the SBR influent (Calli et al. 2003; Pant and Adholeya 2007), which might be the main reason complete COD removal was not possible in the SBR, and further physical or chemical deep treatment is necessary.

Conclusions

The excellent wastewater treatment performance of CSTR–EGSB–SBR fed with cellulosic ethanol production wastewater was closely related to the diverse microbial communities. *Lactobacillus* and *Prevotella* observed in the CSTR contributed to VFAs production. In the EGSB reactor, acetate-utilizing methanogens belonging to *Methanosaetaceae* were identified. Microorganisms associated with the ability to degrade cellulose, hemicellulose, and other biomass-derived organic carbons were observed in the CSTR, EGSB, and SBR. The present study identified different functional microbial communities responsible for the degradation of compounds contained in the cellulosic wastewater and will facilitate development of a suitable system for treatment of cellulosic ethanol production wastewater.

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