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RESEARCH ARTICLE

Performance of CSTR–EGSB–SBR system for treating sulfate-rich cellulosic ethanol wastewater and microbial community analysis

Lili Shan[1] · Zhaohan Zhang[1] · Yanling Yu[1] · John Justo Ambuchi[1] · Yujie Feng[1]

Abstract Performance and microbial community composition were evaluated in a two-phase anaerobic and aerobic system treating sulfate-rich cellulosic ethanol wastewater (CEW). The system was operated at five different chemical oxygen demand (COD)/SO_4^{2-} ratios (63.8, 26.3, 17.8, 13.7, and 10.7). Stable performance was obtained for total COD removal efficiency (94.5%), sulfate removal (89.3%), and methane production rate (11.5 L/day) at an organic loading rate of 32.4 kg COD/(m³·day). The acidogenic reactor made a positive contribution to net VFAs production (2318.1 mg/L) and sulfate removal (60.9%). Acidogenic bacteria (*Megasphaera*, *Parabacteroides*, unclassified *Ruminococcaceae* spp., and *Prevotella*) and sulfate-reducing bacteria (*Butyrivibrio*, *Megasphaera*) were rich in the acidogenic reactor. In the methanogenic reactor, high diversity of microorganisms corresponded with a COD removal contribution of 83.2%. Moreover, methanogens (*Methanosaeta*) were predominant, suggesting that these organisms played an important role in the acetotrophic methanogenesis pathway. The dominant aerobic bacteria (*Truepera*) appeared to have been responsible for the COD removal of the SBR. These results indicate that dividing the sulfate reduction process could effectively minimize sulfide toxicity, which is important for the successful operation of system treating sulfate-rich CEW.

Keywords Cellulosic ethanol wastewater · Sulfate · Two-phase anaerobic digestion · Aerobic treatment · High-throughput sequencing

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Introduction

Ethanol produced from cellulosic materials as an alternative fuel has been attracting worldwide attention because of the abundance of lignocellulosic resources. However, large quantities of wastewater from cellulosic ethanol production pose serious environmental concerns because of its quality [high chemical oxygen demand (COD), color, organic priority pollutant content, biochemical oxygen demand (BOD), and the presence of sulfate resulting from dilute sulfuric acid pretreatment], and considerable technical requirements are required to treat sulfate-rich cellulosic ethanol wastewater (CEW) (Kim and Lee 2002; Vohra et al. 2014; Wilkie et al. 2000).

Wastewaters containing sulfate are generally treated by physicochemical and biological methods. Although physicochemical methods are effective, their usage is restricted owing to their relatively high cost, energy demand, and requirement for a separate system and appropriate disposal of the solid phase (Lens et al. 1998). Biological sulfate removal from wastewaters is a well-known process (Lens and Kuenen 2001), but the production of odorous sulfide during the sulfate reduction process can cause severe disturbance of the methanogenesis process and, in extreme cases, complete performance failure (Lens and Kuenen 2001; Lens et al. 1998). However, the produced sulfide can be isolated from the methane production process by using a two-phase anaerobic digestion with the initial phase for sulfate reduction and the later for

methanogenesis (Reis et al. 1988). The sulfide produced can be stripped directly in the first phase or between the two phases because the main sulfide in the acidification reactor exists as gaseous H_2S (about pH 5.0 to 6.5) (Lens et al. 1998). Sulfate reduction in the acidogenic phase had been considered in two-phase anaerobic digestion systems treating ethanol wastewater from sugar-based and starch-based feedstocks (Choeisai et al. 2014; Reis et al. 1988; Shin et al. 1992). However, few studies have investigated the use of this process to treat sulfate-rich CEW.

Anaerobic digestion of wastewater containing sulfate is a multi-stage biochemical process in which complex organic matter undergoes hydrolysis, acidification, methanogenesis, and sulfate reduction (Fox and Pohland 1994; Lens et al. 1998). These reactions rely on a series of microorganisms including fermentative acidogenic bacteria, sulfate-reducing bacteria (SRB), and methanogens (Fox and Pohland 1994; Lens et al. 1998). When these groups appear in a mixed anaerobic culture, there is nutrient competition between SRB and methanogens with the SRB prevailing for a higher energy profit (Lens et al. 1998; Percheron et al. 1997). It has been reported that two-phase anaerobic digestion minimizes this competition in the desulfurization process and enhances stability and performance by providing favorable environments (nutritional and pH requirements) for SRB and methanogens (Lens et al. 1998). However, little information is available about the microbial communities involved in a two-phase anaerobic process for the treatment of ethanol wastewater (Shan et al. 2015).

This study was conducted to assess the performance and microbial community composition in a two-phase anaerobic and aerobic system that consisted of a continuous stirred tank reactor (CSTR), expanded granular sludge bed (EGSB) reactor, and sequencing batch reactor (SBR) treating sulfate-rich CEW. The combined system was operated at five different COD/SO_4^{2-} ratios (63.8, 26.3, 17.8, 13.7, and 10.7), and COD, sulfate, liquid fermentation products, and biogas products were investigated. High-throughput sequencing based on Illumina Miseq system was also applied to study the functional microbial populations in the combined system. The results presented herein will provide valuable information for the development of a highly available system for treatment of sulfate-rich CEW.

Materials and methods

Characteristics of the sulfate-rich CEW

The combined system was fed with the wastewater from a cellulosic ethanol plant (Heilongjiang province, China) using corn stover as feedstock. The characteristics of the sulfate-rich CEW are presented in Table 1. Corn stover was pretreated with dilute sulfuric acid, which resulted in high sulfate concentration in the wastewater. Other important characteristics of sulfate-rich CEW include high COD, BOD, low pH, and color of dark brown.

Wastewater treatment system

A combined system of CSTR–EGSB–SBR was applied for sulfate-rich CEW treatment. The schematic of the system configuration was provided in our previous study (Shan et al. 2015). As stated previously, the combined system for treating CEW was operated for 139 days. The influent of this system was changed to synthetic sucrose wastewater prior to the sulfate-rich CEW for 8 months. The combined system was then fed by sulfate-rich CEW and operated for 3 months. In the initial period, a synthetic sucrose wastewater was introduced into the system for stabilized performance (98.5% COD removal) until the organic loading rate was 32.4 kg $COD/(m^3 \cdot day)$ (COD 13,497.2 mg/L). Subsequently, mixing wastewater was continuously provided by increasing the ratio (20%, 40%, 60%, 80%, 100%) of diluted sulfate-rich CEW, but at a constant organic loading rate, thus leading to different COD/SO_4^{2-} ratios. The influent characteristics of the diluted sulfate-rich CEW (100% ratio) are presented in Table 1. The system was operated under five periods depending on the COD/SO_4^{2-} ratios: 63.8, 26.3, 17.8, 13.7, and 10.7 (runs 1, 2, 3, 4, and 5; Table 2). Sludge samples were collected for microbial diversity analysis when the system stabilized in the last period.

Microbial community analysis

The microbial communities of the combined system were analyzed by high-throughput sequencing. Genomic DNA was isolated from all sludge samples using a E.Z.N.A.™ Soil DNA Kit (D5625-01; Omega Bio-tek Inc., USA). The extracted DNA was investigated using electrophoresis on 1% agarose gel and quantified using a Qubit 2.0 spectrophotometer (Invitrogen, USA). The bacterial 16S rDNA PCR and sequencing was performed using 515F (5′-GTGC CAGCMGCCGCGG-3′) and 907R (5′-CCGT CAATTCMTTTRAGTTT-3′) primers targeting the variable region V4–V5. Archaeal 16S rDNA nested PCR and sequencing were performed using two pairs of PCR primers, which were 340F (5′-CCCTAYGGGGYGCASCAG-3′) and 1000R (5′-GGCCATGCACYWCYTCTC-3′) and 349F (5′-GYGCASCAGKCGMGAAW-3′) and 806R (5′-GGAC TACVSGGGTATCTAAT-3′). After the PCR and purification process of PCR products, the Miseq sequencing was done using an Illumina Miseq platform for sequencing. More than 10,000 sequences with 400–500 bp length were obtained from each sample. Operational taxonomic units (OTUs) were assigned at a 97% similarity threshold (UCLUST v1.1.579), and the OTUs were regarded as relating to the genus.

Table 1 Characteristics of the sulfate-rich CEW

Parameter	Raw	Influent[a]
Chemical oxygen demand (COD) (mg/L)	78,487.0 ± 5,362.8	13,497.2 ± 912.9
Biochemical oxygen demand (BOD) (mg/L)	27,530.0 ± 5,761.1	4,776.0 ± 946.2
Total nitrogen (TN) (mg/L)	678.4 ± 32.7	153.0 ± 5.5
Total phosphorus (TP) (mg/L)	258.1 ± 20.5	31.1 ± 2.3
Sulfate (mg/L)	7,137.0 ± 217.3	1,260.5 ± 44.7
Ethanol (mg/L)	100.7 ± 15.1	14.7 ± 2.1
Acetate (mg/L)	5,790.3 ± 697.5	422.3 ± 51.2
Propionate (mg/L)	308.5 ± 100.3	81.9 ± 17.7
Butyrate (mg/L)	131.2 ± 33.6	21.3 ± 4.0
Valerate (mg/L)	55.2 ± 15.4	6.3 ± 1.4
pH	4.4 ± 0.2	6.1 ± 0.7[b]
Color	Dark brown	Dark brown

[a] Diluted raw wastewater was fed as the final influent of the system

[b] Influent pH of the system was adjusted by the addition of $NaHCO_3$

Taxonomic unit classification of the sequences of each sample was carried out using the RDP Classifier to provide taxonomic assignments, based on Bergey's taxonomy. Community estimators were calculated and analyzed by mothur (http://www.mothur.org/), including richness estimators: abundance-based coverage estimator (ACE) index, Chao1 index, Simpson index, and Shannon index.

The Illumina Miseq sequencing data was deposited in the NCBI Sequence Read Archive under project SRP076590.

Analytical methods

COD of influent and effluent in the reactors were measured by standard methods for the examination of water and wastewater (APHA et al. 2005). The total COD removal and COD removal contribution were calculated on basis of the influent of the system, while removal efficiency of reactor was calculated on the basis of the influent and effluent of each individual reactor. pH, biogas composition (CH_4 and CO_2), and volatile fatty acids (VFAs) were determined as previously described (Shan et al. 2015). Net VFA production was calculated on the basis of the influent of the system. After filtrating with 0.45 μm filter membrane, sulfate in effluents were analyzed by an ion chromatography (ICS-3000; Dionex, USA). Sulfide concentration was measured using Sure-Flow™ combination silver/sulfide electrodes (Berner 1963).

Results and discussion

Performance of the combined system

The performance of the two-phase anaerobic and aerobic system at a constant COD of 13,497.2 mg/L with different COD/SO_4^{2-} ratios is shown in Fig. 1. Overall, stable operation of the combined system was obtained with constant COD removal. Table 2 shows the performance of the combined system at steady states. As the influent ratio of sulfate-rich CEW increased (runs 1, 2, 3, 4, 5), COD removal efficiency was generally stable throughout the runs, only slightly decreased from 98.2% (run 1) to 94.5% (run 5) (Fig. 1a). This was likely because the available substrate was limited. When complex substrates were used in the system, an initial hydrolysis and fermentation process was necessary to degrade complex macromolecules to simpler ones and transform them to acetate and other readily biodegradable substrates (Montpart et al. 2015) that would be further utilized by methanogens. It should be noted that the hydrolysis and fermentation process of complex substrate was the rate-limiting step (Jimenez et al. 2014).

Each reactor of the system contributed to COD removal, but the potential was different. COD removal efficiency in the acidogenic reactor (CSTR) was not expected to be high (6.0%), while CSTR was shown to achieve high hydrolysis and fermentation ability (Demirel and Yenigün 2002). A large
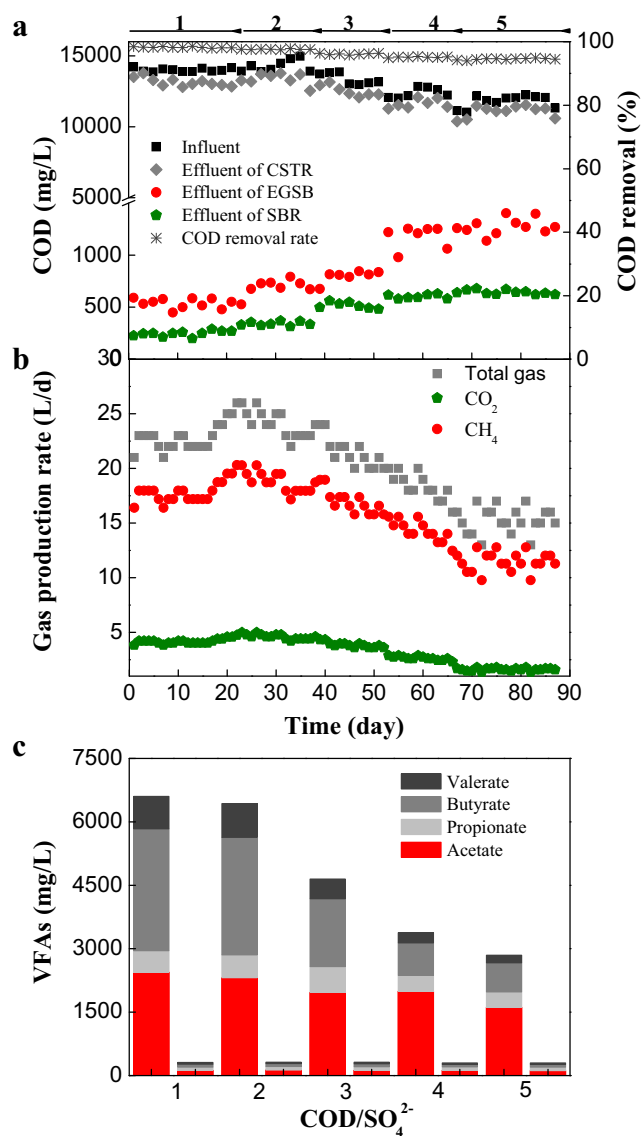
Table 2 Operational and performance characteristics of the two-phase anaerobic and aerobic system

Run	1	2	3	4	5
CEW ratio (%)	20	40	60	80	100
Sulfate (mg/L)	211.7 ± 14.9	512.4 ± 26.9	757.8 ± 23.3	982.8 ± 74.6	1260.5 ± 44.7
COD/SO_4^{2-}	63.8	26.3	17.8	13.7	10.7
COD removal (%)	98.2 ± 0.2	97.6 ± 0.1	96.1 ± 0.2	95.1 ± 0.2	94.5 ± 0.3
Sulfate removal (%)	86.0 ± 3.1	90.6 ± 1.5	87.0 ± 2.0	87.3 ± 1.8	89.3 ± 1.2

Fig. 1 Performance of the two-phase anaerobic and aerobic system: **a** COD, **b** gas production rate, **c** VFAs. *Left column* is CSTR; *right column* is EGSB

amount (2318.1 mg/L) of net VFA production was obtained in run 5 (Fig. 1c), suggesting that they constituted the majority of soluble products. It was likely that acidogenic bacteria (AB) developed in the CSTR. The VFAs gradually decreased with COD removal efficiency throughout the experiment, indicating that AB developed in the system to convert complex substrates to acidified products. When the sulfate-rich CEW was used as the influent of the system (run 5), acetate, butyrate, propionate, and valerate accounted for an average of 56.7, 24.2, 12.7, and 6.4%, respectively, indicating that the butyrate-type fermentation pathway was obtained in the CSTR. In addition, a considerable amount (1615.7 mg/L) of acetate in the CSTR might promote the growth of acetate-utilizing methanogens found in the subsequent EGSB reactor (Ahring et al. 2001).

Compared to the CSTR, the methanogenic reactor (EGSB) made a positive contribution to COD removal (83.2%) since most of the VFAs produced in the acidogenic phase were converted to methane. Figure 1b shows biogas products in the EGSB reactor throughout the experiment. As the influent ratio of sulfate-rich CEW increased, the total gas production rate gradually decreased with VFA production in the CSTR. In run 1, the biogas production rate was 22.9 L/day and typically consisted of 78.1% methane, 18.3% carbon dioxide, and 3.6% other gas (Fig. 1b). In run 5, the biogas production rate decreased to 15.2 L/day and the ratio of methane was 75.2%. These findings indicate that the available substances generated from hydrolysis and fermentation processes for methanogens were reduced. Moreover, sulfate stimulated the growth of SRB, which might outcompete methanogens for substrates (H_2 and acetate) (discussed later) (Percheron et al. 1997).

The acidogens and methanogens were successfully separated in the two reactors, and CSTR–EGSB anaerobic digestion was confirmed to perform efficiently. However, the effluent of CSTR–EGSB still had a high COD (1275.8 mg/L); hence, a SBR was utilized for aerobic post-treatment. The COD removal efficiency of the SBR was not expected to be high (49.3%) in run 5 for the low B/C ratio (0.15) (Fig. 1a). In summary, relatively stable operation in runs 1–5 was achieved with overall COD removal efficiency of 94.5–98.2% at OLR of 32.4 kg COD/(m^3·day).

The sulfate reduction of the combined system

Dilute sulfuric acid pretreatment was used in the cellulose conversion processes, which resulted in high sulfate concentration in the CEW. The results of sulfate removal in the CSTR–EGSB–SBR system, which are shown in Fig. 2a, indicated that the CSTR made a positive contribution to sulfate removal. The sulfate concentration of effluent was gradually increased throughout the experiment. Finally, the total sulfate removal efficiency in run 5 was 89.3%, and sulfate removal in the CSTR accounted for 60.9%. The sulfate reduction process is carried out by a group of microorganisms considered SRB. It should be noted that AB had a close relationship with SRB in the CSTR (Kalyuzhnyi et al. 1998); specifically, organic materials were degraded by AB to acidified products that could be subsequently utilized by SRB for sulfate reduction.

The biological sulfate reduction process resulted in sulfide production under anaerobic conditions. The sulfide in the CSTR was increasing with reducing the COD/SO_4^{2-} ratio, and the trend in the EGSB reactor showed some difference, which corresponded with sulfate removal in anaerobic reactors (Briones et al. 2009). The sulfate removal in the CSTR was relatively smooth in the initial period (runs 1 and 2) and gradually increased during runs 3–5, while the sulfate removal in the EGSB reactor was found to fluctuate slightly throughout the runs, which probably resulted in a change of the sulfide.
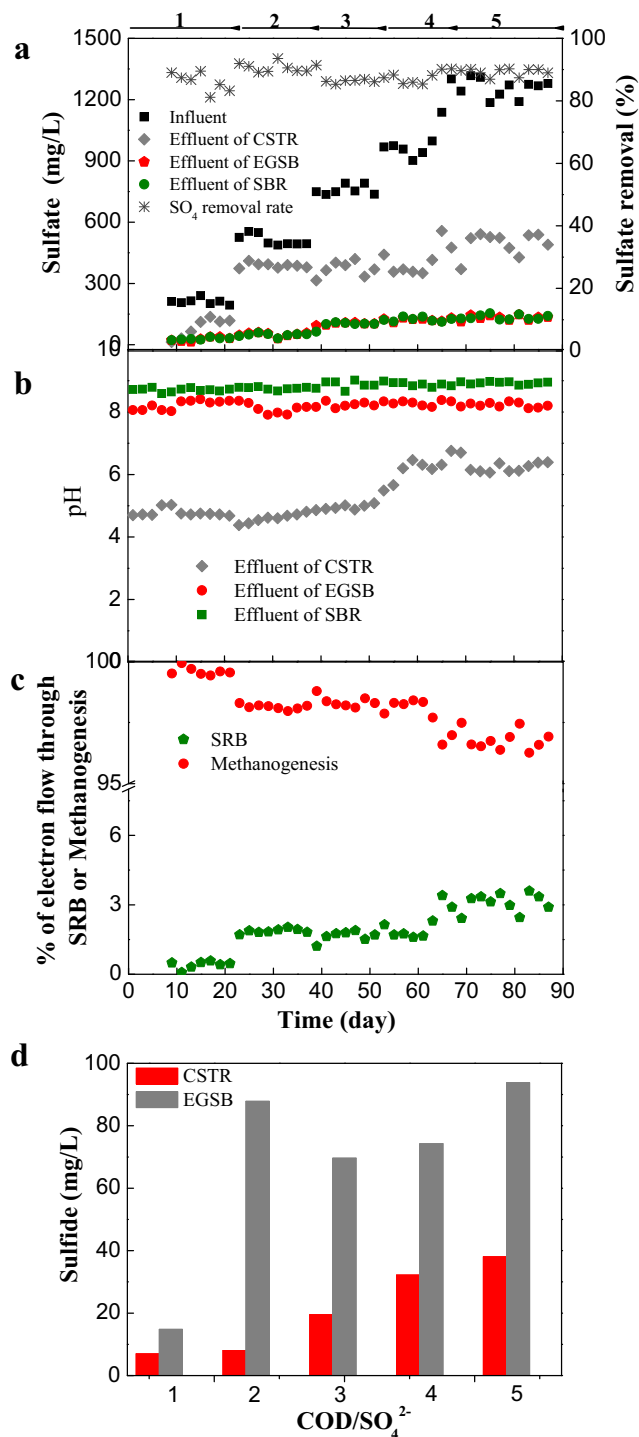
Fig. 2 Sulfate reduction of the two-phase anaerobic and aerobic system: **a** sulfate, **b** pH, **c** % electron flow through SRB or methanogenesis, **d** sulfide

The effluent sulfide concentration of the CSTR significantly underestimated the theoretical amount of sulfate reduction. It should be noted that sulfide volatilization was dependent on solution pH. The determined effluent pH of the CSTR was 6.3 (Fig. 2a), so the main form of sulfide was estimated as hydrogen sulfide (Lens et al. 1998), and low sulfide concentrations

(38.1 mg/L) were detected in the CSTR (Fig. 2d). While in the EGSB reactor, the determined effluent pH of EGSB was 8.2, so the main form of sulfide was estimated to be dissolved sulfide (Lens et al. 1998). When the sulfate-rich CEW was used as the influent (run 5) in the system, sulfide in the effluent of the EGSB was 93.8 mg/L. The reported concentrations at which total dissolved sulfide inhibited methanogenesis varied considerably and might have been affected by factors (microbial composition and substrate) in each case (Lens et al. 1998). Thus, no definite conclusion could be drawn about the reactor inhibition.

However, during the anaerobic digestion process, the possibility of competition between methanogens and SRB always exists. To evaluate if either the methanogenic or sulfidogenic pathway was functioning in the EGSB reactor, the percent electron flow was calculated as previously described (Isa et al. 1986). As shown in Fig. 2c, decreasing the COD/SO_4^{2-} ratio increased the percent electron flow by the SRB. However, methanogenesis accounted for 96.8–99.6% of the total electron flow throughout the experiment. These results indicate that electron flow for methanogenesis was dominant in the EGSB reactor, and that methane production was more resilient than in a previously investigated single reactor in response to an increase in sulfate load (Briones et al. 2009). It is likely that the two-phase system reduced sulfide toxicity and stabilized the performance.

Microbial community analysis

The biological treatment of organic wastewater containing sulfate relies on a complex microbial community (Fox and Pohland 1994; Lens et al. 1998). Evaluation of microbial behavior is useful for enhancing system performance. The microbial community composition of the combined system was analyzed by high-throughput sequencing based on an Illumina Miseq system. The Shannon, Simpson, ACE, and Chao1 indices, as well as the OTUs were used to estimate the microbial diversity and richness (Table 3). For bacteria, samples in the EGSB had a higher Shannon and lower Simpson index when compared with other reactors, indicating a much higher diversity of bacterial species. This could be interpreted as a positive contribution of the EGSB reactor to COD removal (Pholchan et al. 2010). Moreover, samples had greater bacterial richness in terms of more OTUs as well as higher ACE and Chao richness estimators than other reactors because of the inhomogeneous environment in the EGSB. These results indicated that the EGSB reactor led to a significant improvement of the performance of the combined system.

Bacterial diversity in the CSTR

As shown in Fig. 3a, bacterial sequences affiliated with *Firmicutes* (58.9%) and *Bacteroidetes* (38.3%) were dominant

Table 3 Estimators for evaluation of microbial community diversity and richness

Samples	OTUs	ACE	Chao1	Shannon index	Simpson index	Coverage (%)
Bacteria						
CSTR	114	119.45	119.5	2.87	0.12	99.91
EGSB-up	308	394.69	366.52	2.57	0.29	99.42
EGSB-down	325	399.44	400.48	3.60	0.07	99.54
SBR	295	355.35	375.16	2.30	0.39	99.62
Archaea						
EGSB-up	303	1009.56	688.92	1.56	0.53	98.17

in the CSTR. At the genus level, dominant populations included *Megasphaera*, *Parabacteroides*, unclassified *Ruminococcaceae* spp., and *Prevotella*, with proportions of 27.9, 22.3, 15.7, and 8.8%, respectively (Fig. 3c). Species from all of the aforementioned genera were members of fermentative AB, which have been confirmed to have the ability to produce acids from various sugars and even some complex polysaccharides such as xylan, cellulose, and hemi-celluloses (Guo et al. 2015; Hino et al. 1991; Huws et al. 2011). The existence of those populations in the CSTR likely made a positive contribution to treatment of sulfate-rich CEW. Moreover, the major fermentation products of *Parabacteroides* and *Prevotella* are acetate and succinic acid (Tan et al. 2012; Shen et al. 2013), indicating that these organisms may have been responsible for the high (56.7%) acetate production. Species of *Megasphaera* and *Butyrivibrio* (3.6%) have been reported to be important to butyrate production (Hino et al. 1991), which might correspond with the butyrate-type fermentation pathway in the CSTR.

In addition, species affiliated with *Paludibacter* (4.2%), *Butyrivibrio* (3.6%), and *Oscillibacter* (3.3%) in the CSTR were reported enrichments of sulfate reduction systems under acidic conditions (Emery et al. 1957; Lee et al. 2013; Sánchez-Andrea et al. 2013), and *Paludibacter* spp. was often accompanied by the SRB in sulfate reduction systems (Zheng et al. 2014). Sulfate reducers in the CSTR included members affiliated with *Butyrivibrio* and *Megasphaera*. Previous studies demonstrated that *Butyrivibrio* and *Megasphaera* species showed an appreciable ability to utilize inorganic sulfate (Emery et al. 1957; Hino et al. 1991). The presence of those populations might play an active role in the sulfate reduction of the CSTR, which made a positive contribution to sulfate removal (60.9%). In theory, conversion of 1 mol of sulfate requires 0.67 mol of COD or electron donors (Lens et al. 1998). Hydrogen is an attractive electron donor for sulfate reduction due to its low free energy (Lens et al. 1998). Although hydrogen production was not determined in the CSTR, the presence of *Megasphaera*, unclassified *Ruminococcaceae* spp., and *Prevotella* species, which are known to be associated with the biohydrogenation process (Hino et al. 1991; Huws et al. 2011), suggested that hydrogen provided protons in the sulfate reduction process, resulting in a pH increase in the CSTR (Fig. 2b).

Microbial diversity in the EGSB reactor

We previously analyzed the microorganisms in the EGSB reactor from top to bottom (as level A–D) and found that the diversity and abundance of most bacterial and all archaeal species gradually increased from bottom to top (Shan et al. 2015). Therefore, the sludge samples were collected at up (level B) and down (level D) of the EGSB reactor for bacteria analysis in this study, while only up (level B) samples were collected for archaea analysis (Fig. 1S).

The dominant bacterial phyla identified in the EGSB (up, down) were affiliated with *Firmicutes* (9.5%, 7.7%), *Thermotogae* (7.5%, 10.3%), *Proteobacteria* (5.3%, 13.1%), *Bacteroidetes* (4.3%, 6.1%), *Synergistetes* (5.3%, 7.1%), *Hyd24-12* (52.8%, 19.9%), and others (15.5%, 35.9%) (Fig. 3a). *Thermotogaceae* and *Syntrophomonadaceae*, which were affiliated with the phylum *Thermotogae* and *Firmicutes*, respectively, were found in the EGSB. Species belonging to *Thermotogaceae* and *Syntrophomonadaceae* were reported as butyrate degradation bacteria and had the ability to oxidize butyrate to carbon dioxide/hydrogen and acetate (Lykidis et al. 2011; Stams et al. 2005). This might account for the large reduction of butyrate (624.3 mg/L) in the EGSB reactor. Moreover, *Thermotogaceae* (7.5%, 10.3%) and *Syntrophomonadaceae* (1.7%, 2.9%) occupied a significant proportion of the EGSB reactor, which might correspond to the butyrate-type fermentation pathway of the CSTR (Fig. 1c).

Alphaproteobacteria, *Gammaproteobacteria*, and *Deltaproteobacteria* belonging to the phylum *Proteobacteria* were also observed in the EGSB reactor, but only *Deltaproteobacteria* occupied a significant proportion (85.0%, 80.4%) of both the upper and lower layer of the EGSB reactor. Moreover, the primary genera belonging to *Deltaproteobacteria* were affiliated with *Syntrophorhabdus*, *Syntrophobacter*, and unclassified *Desulfuromonadales* species, with proportions of (51.1%, 36.0%), (26.6%, 36.1%), and (8.0%, 10.3%), respectively (Fig. 3c). *Syntrophobacter* species were described as propionate- SRB in physiology and phylogenetics, which used propionate as an electron donor to reduce sulfate (Stams et al. 2005). Correspondingly, propionate of 280.5 mg/L was reduced in the EGSB reactor (Fig. 1c). Recent studies indicate that members of the

Fig. 3 Microbial community distribution and relative abundance of the two-phase anaerobic and aerobic system: **a** bacteria at the phylum level, **b** archaea at the genus level, **c** bacteria at the genus level. Phyla and genera making up less than 1% of the total sequences in each sample were classified as others
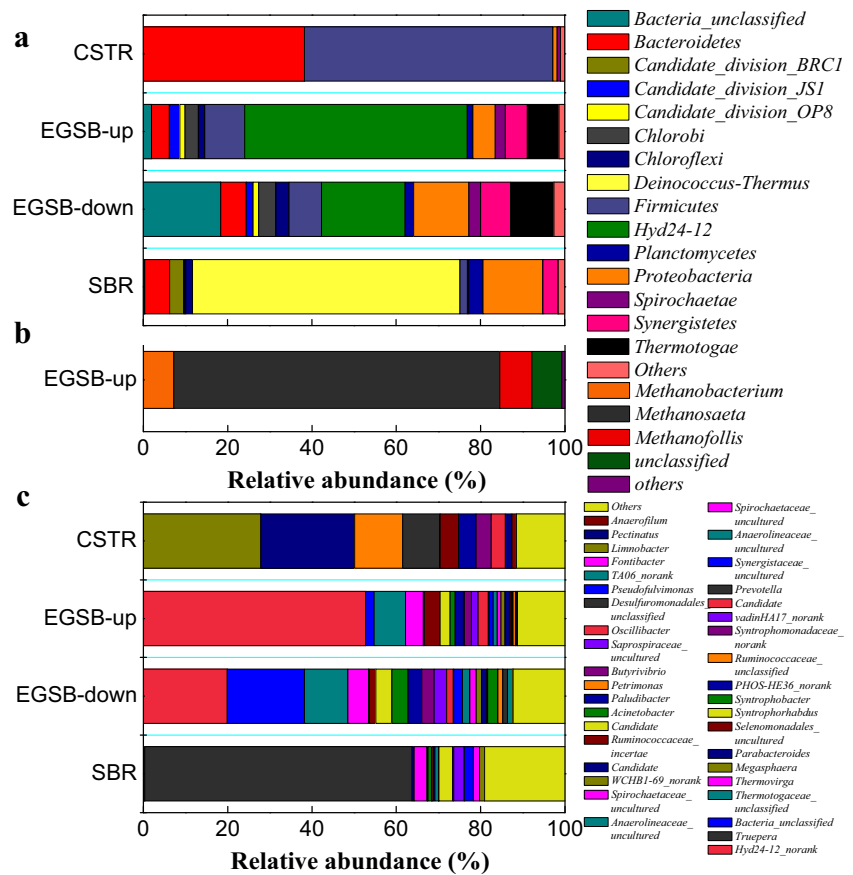
Desulfuromonadales become highly active when acetate is available for oxidation, and that they are also capable of using hydrogen as electron donor (Greene et al. 2009). Moreover, *Thermovirga* species (4.3%, 4.9%) affiliated with *Synergistetes* are anaerobic SRB that utilize organic acid as a carbon source and electron donor (Göker et al. 2012). *Syntrophobacter*, *Thermovirga*, and unclassified *Desulfuromonadales* species detected in the EGSB reactor might facilitate the removal of sulfate from sulfate-rich CEW. The presence of SRB could outcompete methanogenesis for substrates (H_2 and acetate) (Percheron et al. 1997); however, as previously described, methanogenesis remained an important route for electron flow in the EGSB reactor (Fig. 2c).

The archaeal phyla *Euryarchaeota* and *Crenarchaeota* were observed in the EGSB reactor, and the archaeal genera identified in the EGSB (up) were affiliated with *Methanosaeta* (77.3%), *Methanofollis* (7.6%), *Methanobacterium* (7.2%), unclassified archaea (7.1%), and others (0.7%) (Fig. 3b). *Methanosaeta*, which belongs to the phylum *Euryarchaeota* and is considered to be acetoclastic methanogens, was observed in typical granules of the two-phase anaerobic system (Demirel and Scherer 2008). A considerable amount of acetate was found in the CSTR, which might indicate that

Methanosaeta was dominant in the EGSB reactor. The genera *Methanofollis* and *Methanobacterium* utilize hydrogen or formic acid as substrate to produce methane (Demirel and Scherer 2008). However, when hydrogen-utilizing SRB are present in an anaerobic reactor, hydrogenotrophic SRB have an advantage (substrate affinity, growth rate, and cell yield) over hydrogenotrophic methanogens (Lens et al. 1998). This could further indicate that acetoclastic methanogenesis was dominant in the EGSB reactor. These methanogens made a positive contribution to methane production.

Bacterial diversity in the SBR reactor

The dominant bacterial phyla identified in the SBR were affiliated with *Deinococcus–Thermus* (63.5%), *Proteobacteria* (14.2%), *Bacteroidetes* (5.9%), and *Synergistetes* (3.5%) (Fig. 3a). *Truepera* affiliated with *Deinococcus–Thermus* was observed at high relative abundance (63.5%) in SBR. This population was also observed at high abundance in compost-derived enrichments on a purified hemi-cellulose fraction, wheat arabinoxylan (Eichorst et al. 2014). These findings implied that the presence of *Truepera* served a vital function in aerobic degradation of this wastewater. In addition, *Limnobacter* species

(1.2%) belonging to the phylum *Proteobacteria* were described as aerobic sulfur-oxidizing bacteria (Wang et al. 2012) and were able to oxidize sulfide to sulfate. This might explain the slight increase in sulfate concentration in the effluent of the SBR (Fig. 2a). *Thermovirga* (3.1%) species affiliated with *Synergistetes* are considered anaerobic SRB, but were found in this aerobic reactor. These findings indicate that sulfate reduction occurred in the deeper, anoxic microenvironment of the biofilm and was spatially separated from sulfide oxidation (Kühl and Jørgensen 1992). The presence of *Thermovirga* species in the SBR might be attributed to the EGSB effluent, in which *Thermovirga* was observed (Fig. 3c). Sulfate reduction and sulfide oxidation in the aerobic reactor led to the relatively smooth sulfate concentration in the effluent of the SBR (Fig. 2a).

A two-phase anaerobic and aerobic system was used for treatment of sulfate-rich CEW. During long-term operation of the system (runs 1–5), conversion of sulfate-rich CEW into VFAs and sulfate reduction in the first phase (CSTR) of the two-phase anaerobic digestion provided favorable environmental conditions for methanogenesis in the second phase (EGSB), which achieved a high COD removal. Aerobic post-treatment (SBR) was expected to increase COD removal. Analysis of the functional microbial populations revealed that acidogenic bacteria and microorganisms associated with sulfate reduction were rich in the CSTR, while methanogens remained dominant in the EGSB reactor. Therefore, it can be deduced that dividing the sulfate reduction process had significant effects leading to stable operation of anaerobic digestion. The results revealed a highly efficient treatment technique for sulfate-rich CEW that would benefit the development of the cellulosic ethanol industry.

Conclusions

Promising performance of a combined system (CSTR–EGSB–SBR) fed with sulfate-rich CEW was achieved. The total COD removal was 94.5%, the sulfate removal was 89.3%, and the methane production rate was 11.5 L/day. VFA production and sulfate reduction in the CSTR were mainly due to the presence of *Megasphaera*, *Parabacteroides*, unclassified *Ruminococcaceae* spp., *Prevotella*, *Butyrivibrio*, and *Megasphaera*. High bacterial diversity in the EGSB made a positive contribution of COD removal (83.2%), and preponderant methanogens (*Methanosaeta*) resulted in development of an acetotrophic methanogenesis pathway. Aerobic bacteria (*Truepera*) played a vital role in the degradation of anaerobic effluent.

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