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UASB performance and electron competition between methane-producing archaea and sulfate-reducing bacteria in treating sulfate-rich wastewater containing ethanol and acetate

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HIGHLIGHTS

- MPA predominated in COD and electrons utilization in high sulfate situation.
- At HRT of 6 h, methane yield reached 0.23 L/gCOD with COD removal above 80%.
- Methane was generated by *Methanosaeta concilii GP6* with acetate as substrate.
- Sulfate was mainly reduced by *Desulfovibrio* species with ethanol as substrate.
- SRB accounted for 17.6% in bacteria and all belonged to incomplete oxidizers.

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ABSTRACT

To find an appropriate method for sulfate-rich wastewater containing ethanol and acetate with COD/sulfate ratio of 1, a UASB reactor was operated for more than 180 days. The influences of HRT (hydraulic retention time) and OLR (organic loading rate) on organics and sulfate removal, gas production, and electrons utilization were investigated. The sludge activity and microorganism composition were also determined. The results indicated that this system removed more than 80% of COD and 30% of sulfate with HRT above 6 h and OLR below 12.3 gCOD/L d. Further HRT decrease caused volatile fatty acids accumulation and performance deterioration. Except at HRT of 2 h, COD and electron flow were mostly utilized by methane-producing archaea (MPA), and methane yield remained in the range of 0.18–0.24 LCH_4/gCOD. Methane was mainly generated by *Methanosaeta concilii GP6* with acetate as substrate, whereas sulfate was mainly reduced by incomplete-oxidizing *Desulfovibrio* species with ethanol as substrate.

1. Introduction

Anaerobic digestion is widely utilized especially in high strength organic wastewater treatment since it is both cost-effective and environmentally safe. There are many anaerobic digestion processes for sulfate-rich wastewater treatment such as UASB (Lens et al., 1998), expanded granular sludge bed (Dries et al., 1998), membrane reactor (Vallero et al., 2005) and anaerobic fluidized-bed reactor (Kaksonen et al., 2003). These processes are usually efficient in organics removal and methane production with low sulfate concentration. However, the presence of high sulfate in wastewater can cause significant problems resulting from sulfate reduction (Mizuno et al., 1998). In anaerobic treatment processes, SRB (sulfate-reducing bacteria) and MPA (methane-producing archaea, in many literatures called as methane-producing bacteria) always compete for carbon source (Acharya et al., 2008). In sulfate-rich wastewater digestion, SRB often outcompete MPA, and produce corrosive and poisonous sulfide during sulfate reduction (Xu et al., 2012). High level of sulfide is toxic to both MPA and SRB. Its accumulation in the digestion reactors usually causes inhibition effects on organics removal and methane production, and can even result in system failure. Moreover, large quantities of sulfide formation can affect biogas quantity and quality. Consequently, there have been many studies on alleviating the influence of sulfide in anaerobic digestion by using sulfide removal steps and processes, and some researchers have searched for appropriate methods to suppress sulfate reduction and improve organics removal in anaerobic reactors (Aboutalebi et al., 2012).

SRB are anaerobic microorganisms that employ sulfate as an electron acceptor to produce hydrogen sulfide. According to the

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completeness of organics biodegradation during sulfate reduction, SRB are usually classified into two categories: complete oxidizing SRB and incomplete oxidizing SRB. The competition between SRB and MPA in digestion depends largely on the types of substrates and COD/sulfate ratio (Li et al., 1996). SRB can utilize many low molecular weight compounds including butyrate, lactate, propionate, acetate, ethanol and methanol (Nagpal et al., 2000). Normally, SRB have an advantage over MPA during such substrates utilization due to their favorable kinetic properties and thermodynamic conditions (Mizuno et al., 1998). In low COD/sulfate situation, SRB always predominate in carbon source utilization and electron flow transmission, and suppress the activity of MPA (Shin et al., 1997). In a study with ethanol, lactate and glycerol as substrates for anaerobic sulfate reduction, no methane production was observed (Dinkel et al., 2010). In a horizontal-flow anaerobic reactor treating sulfate-rich wastewater with ethanol, acetate, propionate and butyrate as carbon sources, Damianovic and Foresti (2007) found that sulfidogenesis predominated in organics removal and no methane was detected in the biogas.

Although there have been some researches on sulfate reduction with ethanol and acetate as carbon sources, most of them have been focused on how to improve sulfate removal and how to enhance heavy metals removal with sulfide formation during sulfate reduction. Few studies have been done on how to promote organics removal and methane production in high sulfate situation. Ethanol is often considered as an excellent substrate for sulfate reduction since sulfidogenesis always takes predominance in the presence of ethanol in sulfate-rich wastewater digestion. This usually results in acetate accumulation and low methane production. In this research, to find an appropriate method for wastewater containing about 3000 mg/L of sulfate (SO_4^{2-}), 1000 mg/L of ethanol and 1000 mg/L of acetate (about 3000 mg/L of COD in total), a UASB reactor had been run for more than 180 days. The performance of this reactor in COD and sulfate removal, sulfide formation and gas production under different HRT (hydraulic retention time) and OLR (organic loading rate) was studied. The balance of COD and sulfate conversion during digestion was also investigated. According to methane and sulfide production in this reactor, the competition between MPA and SRB in electron flow utilization at different HRT was analyzed. To elucidate the pathways of COD and sulfate removal and conversion, SMA (specific methanogenic activity) and SSA (specific sulfidogenic activity) of the granular sludge in this reactor were determined with batch experiments, and the composition of microorganisms was analyzed with gene cloning method. These results were used to clarify the main digestion pathways of ethanol and acetate. This reactor had shown high performance with regard to both organics removal and methane production.

2. Methods

2.1. Reactor

The UASB reactor used in this study is shown in Fig. 1. This reactor was made of organic glass with an internal diameter of 100 mm. It had a reacting zone with height of 0.8 m and volume of 6 L. The wastewater was pumped from an influent tank with effective volume of 70 L. Heated water was supplied with a water circulation heater to the outer layer of the reactor to keep this reactor with relatively stable temperature of 35 ± 1 °C.

This reactor was inoculated on Oct. 10, 2011, with 3 L mesophilic granular sludge from a full-scale UASB reactor treating food manufacturing wastewater in Miyagi, Japan. With a starting-up HRT of 48 h, more than 85% of COD was removed after 15 days operation. The granular sludge was kept well during the whole operation, and the average diameter and sedimentation velocity of the granules were about 1.8 mm and 96.5 m/h, respectively.

2.2. Wastewater composition

This research was done to study the feasibility of anaerobic treatment to actual wastewater from a chemical industry plant. According to the main composition of the real wastewater, synthetic wastewater was made in laboratory. This wastewater contained about 1000 mg/L acetate, 1000 mg/L ethanol and 3000 mg/L sulfate, and had COD/sulfate ratio of approximately 1. Sodium sulfate was used to supply sulfate in the wastewater. $NaHCO_3$ dosage was controlled at 3000 mg/L except at the starting-up HRT of 48 h with dosage of 1500 mg/L. The other constituents in the synthetic wastewater were as follows: NH_4Cl, 850 mg/L; KCl, 750 mg/L; K_2HPO_4, 250 mg/L; KH_2PO_4, 100 mg/L; $MgCl_2{\cdot}6H_2O$, 125 mg/L; $CaCl_2$, 15 mg/L; $FeCl_2{\cdot}4H_2O$, 42 mg/L; $CoCl_2{\cdot}6H_2O$, 4.2 mg/L and $NiCl_2{\cdot}6H_2O$, 4.2 mg/L.

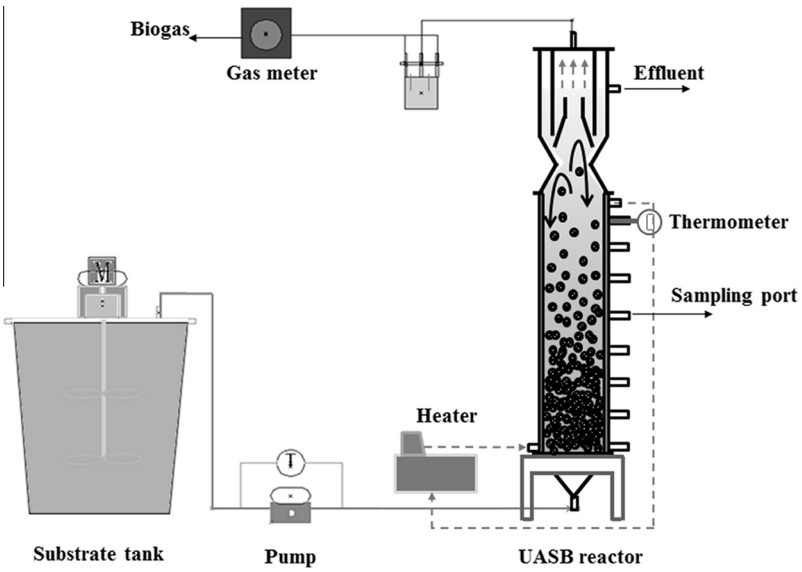

Fig. 1. Schematic diagram of the granular sludge UASB.

2.3. Analytical methods

For sulfate, ethanol, acetate, dissolved sulfide and dissolved COD, the samples were filtered through 0.45 μm polyethersulfone membrane before analysis. COD, dissolved sulfide, total sulfide, alkalinity, total suspended solids (TSS) and volatile suspended solids (VSS) were measured with standard methods according to the American Public Health Association method (APHA, 1995). Free sulfide (undissociated H_2S) in the water was calculated by the first stage ionization equilibrium of hydrogen sulfide with water pH and dissolved sulfide concentration (Omil et al., 1995). Acetate, ethanol and other volatile fatty acids (VFA) were analyzed by gas chromatography (GC, Agilent 6890). Sulfate was analyzed by ion chromatography (DIONEX, DX-120). pH was measured with a pH meter (TOA,HM-30V). The biogas production in this reactor was measured with a wet gas meter (SHINAGAWA W-NK-0.5), and was converted to the number at standard state (0 °C, 1 atm). The contents of CH_4, CO_2 and H_2 in the biogas were determined with a gas chromatography (SHIMADZU GC-8). H_2S in the biogas was measured with hydrogen sulfide detecting tubes (Gastec, No. 4H).

2.4. Activity test procedure

On Day 174, some granular sludge was taken out from the reactor. The sludge activity was determined in 120 mL flasks with substrates of 80% H_2 + 20% CO_2, acetate, ethanol and the synthetic wastewater, respectively. After the inoculation with 2 g of wet sludge to each flask, the flasks for $H_2 + CO_2$ were filled with 40 mL nutrient solution, and the other flasks were filled with 80 mL nutrient solution or wastewater. After the flasks were sealed, nitrogen gas was used to purge the air in the upper space, and 1 mL of 8.125 g/L Na_2S was added into each flask to eliminate the oxygen in the nutrient. The initial COD concentration in the flasks with acetate or ethanol was 3000 mg/L. At the beginning, the gas in the flasks with $H_2 + CO_2$ was displaced with pressurized gas of 80% H_2 + 20% CO_2 to get an initial pressure of 1.4 atm. The activity test was conducted at 35 ± 1 °C in a thermostat. During the test, the gas production and composition were measured every 2 h, and the methane production in every flask was determined. In activity tests with the presence of sulfate, 3000 mg/L sulfate was added to the nutrient solution. Except the methane production, the residual sulfate concentration in the flasks was determined every 2 h. Methane production and sulfate reduction at different intervals were converted into COD removal according to stoichiometry, and in this way, the SMA and SSA of the granular sludge with different substrates were obtained.

2.5. Cloning analysis of 16S rDNA gene

On Day 182, the microbial community was analyzed by 16S rDNA gene cloning and sequencing (Theron and Cloete 2000). Genomic DNA was extracted from samples with an Ultra Clean Soil DNA Isolation Kit (MO-BIO). The amplification of 16S rDNA was performed with the primers EUB 8F (Weisburg et al., 1991) and Univ-1500R (Amann et al., 1990) for bacteria and A109F (Grosskopf et al., 1998) and 1059R (Yu et al., 2005) for archaea. Thermal cycling of PCR consisted of 30 s denaturing at 94 °C, 40 s of annealing at 50 °C, and extracting at 72 °C for 1 min with 30 cycles for archaea and 23 cycles for bacteria. The PCR products were firstly purified by Micro Spin™ S-400 HR (Amersham Pharmacia GE, USA). The purified DNA was then cloned with the TOPO TA Cloning® Kit (Invitrogen,USA) and transformed into *Escherichia coli* $DH5_a$ competent cells. Cloned DNA fragments were obtained and spread on plates. After an incubation period of 24 h at 37 °C, the white ones were randomly picked out and transferred to Luria–Bertani broth with another 6 h of continuous incubation. Insert check was performed using vector of M13 primer. The successful ones were used for sequencing. Similarity searches for the assembled sequences were performed using the NCBI Blast search program within the GenBank database (http://www.ncbi.nlm.nih.gov/blast/).

3. Results and discussion

3.1. Organics removal performance

As shown in Figs. 2(a), (b) and 3(a), with the influent COD kept around 3000 mg/L, HRT of this reactor was decreased gradually from 48 to 2 h, and OLR increased from 1.4 to 37.8 gCOD/L d. With HRT above 6 h and OLR below 12.3 gCOD/L d, COD removal rate was steadily maintained in the range of 86.5–90.9%. When OLR was further increased with HRT reduction, COD removal decreased greatly. At OLR of 37.8 gCOD/L d with HRT of 2 h, only approximately 42% of COD was removed. It can also be noticed from Fig. 3(a) that organic removal rate (ORR) increased linearly with OLR below 12.3 gCOD/L d. After this point, the increase rate of ORR slowed down, and ORR reached the highest value of 17.8 gCOD/L d at OLR of 24.7 gCOD/L d. Then ORR decreased to 15.9 gCOD/L d at OLR of 37.8 gCOD/L d.

There was an obvious increase of VFA in the effluent accompanying HRT reduction and OLR increase (Fig. 2(c)). At HRT of 6 h, VFA in the effluent was below 360 mg/L. At HRT of 3 h, it rose above 520 mg/L. The highest VFA levels reached around 1120 mg/L at HRT of 2 h, and for the first time, a quantity of propionate, butyrate and valerate was detected in the effluent. There was also an obvious decrease in the effluent pH from around 7.4 to 6.9 at HRT of 2 h (Fig. 2(d)). At this HRT, the high VFA in the effluent resulted in COD removal deterioration. However, there was still no ethanol detected in the effluent, which indicated ethanol was decomposed or converted completely. Acetate removal in anaerobic digestion is usually taken as the limiting step in COD removal. In the study of Kaksonen et al. (2004) with ethanol and acetate as carbon source for sulfate reduction, over 99% of ethanol was oxidized with HRT ranging from 20.7 to 6.1 h, and further HRT reduction caused acetate accumulation and process failure. In this study, as the HRT was adjusted back to 6 h with OLR around 12.3 gCOD/L d from Day 135, VFA in the effluent decreased gradually. After 10 days of recovery, VFA in the effluent decreased below 400 mg/L (acetate) and this system regained COD removal above 80%. Consequently, this UASB could be run stably at HRT of 6 h with OLR around 12.3 gCOD/L d.

3.2. Sulfate removal and sulfide variation

Although COD removal changed greatly with HRT reduction and OLR increase, the removal rate of sulfate was relatively stable. As shown in Figs. 2(e) and 3(b), sulfate removal was kept in the range of 28.2–42.5%. With OLR ranging from 6.1 to 18.0 gCOD/L d, sulfate removal only varied from 35.9% to 31.2%. Despite the serious acidification at OLR of 37.8 gCOD/L d, this reactor still got sulfate removal of 32.2%. However, sulfate reduction rate (SRR) was almost linearly promoted with OLR increase. As OLR rose from 1.4 to 37.8 gCOD/L d, SRR increased gradually from 0.6 to 11.4 g/L d. This reactor gained SRR of 3.9 g/L d at OLR of 12.3 g/L d with HRT of 6 h, which was near the value of 4.3 g/L d reported by Kaksonen et al. (2004) but much lower than that (6.33 g/L d) achieved by Nagpal et al. (2000) at similar HRT. When this reactor resumed HRT of 6 h and OLR of 12.3 g/L d from Day 135 to 182, sulfate removal was maintained at around 30%. There was no evidence of sulfate removal increasing with time extension. SRB usually competes effectively at low substrate levels (Isa et al., 1986). However, at HRT of 6 h, there was still above 350 mg/L of COD left in the

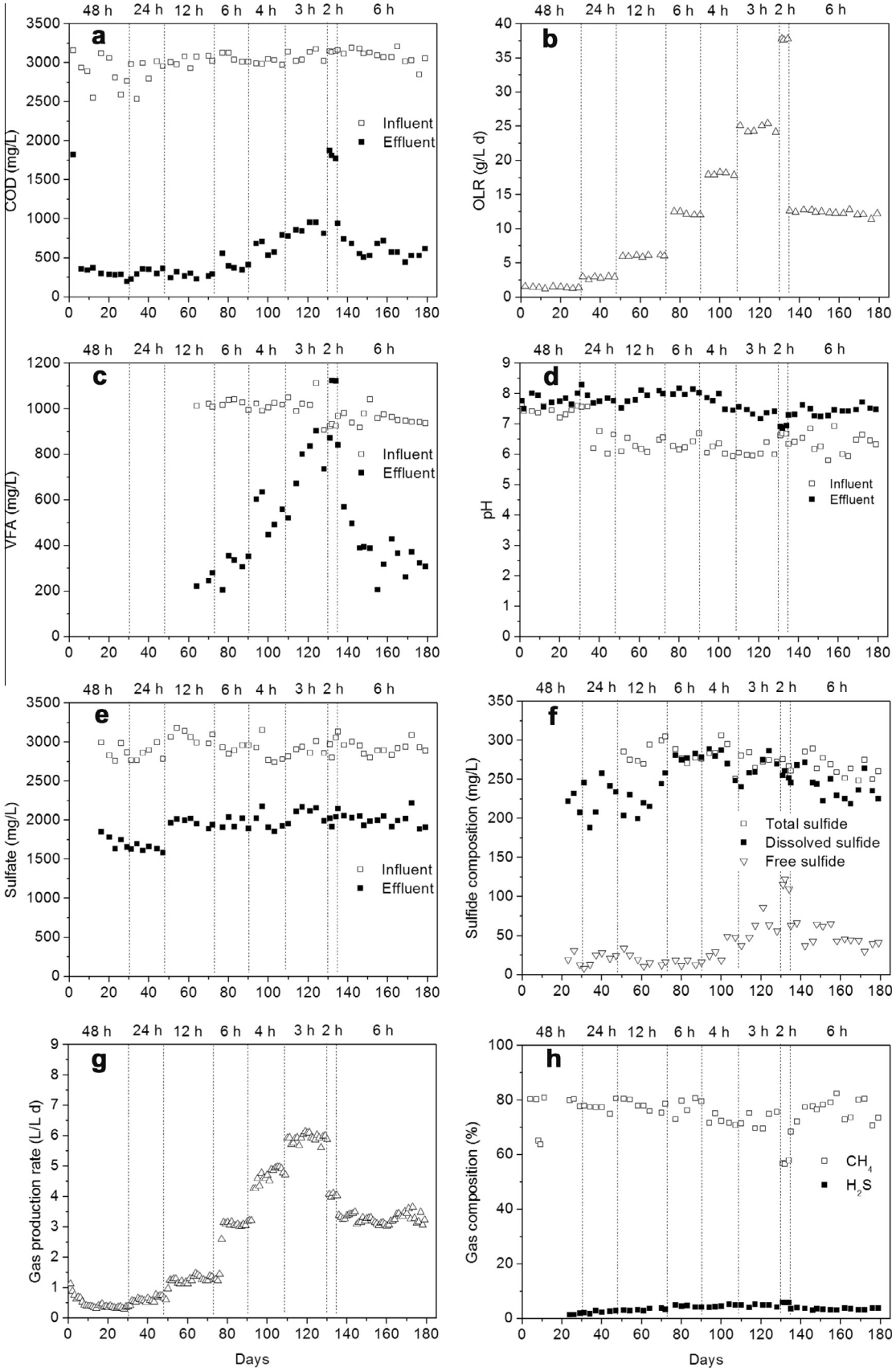

Fig. 2. Overall performance of the UASB in the continuous experiment: (a) COD; (b) organic loading rate (OLR) variation; (c) volatile fatty acids (VFA); (d) pH; (e) sulfate; (f) sulfide composition in the effluent; (g) gas production rate; (h) gas composition.

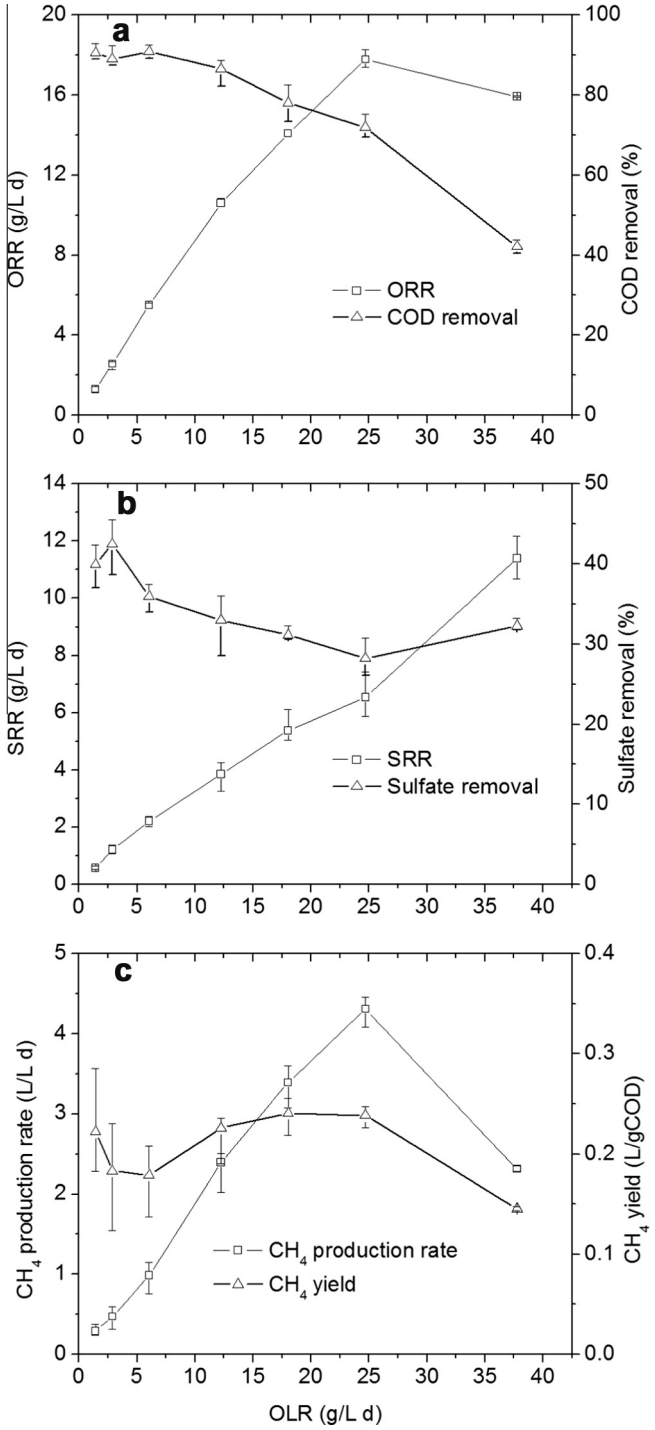

Fig. 3. COD, sulfate removal and methane production variation at different organic loading rates (OLRs): (a) Organic removal rate (ORR) and COD removal percentage; (b) Sulfate reduction rate (SRR) and sulfate removal percentage; (c) methane production rate and yield.

effluent of this UASB (Fig. 2(a)), and SRB could not take predominance at such high substrate levels.

With HRT below 6 h and OLR above 12.3 g/L d, the total sulfide and dissolved sulfide concentration in the effluent remained stable at around 250–300 and 220–290 mg/L, respectively (Fig. 2(f)). However, at low HRTs, pH in the reactor decreased obviously (Fig. 2(d)). According to the equation erected by Omil et al. (1995) on free sulfide calculation, the free sulfide concentration increased greatly, especially when pH decreased drastically with HRT below 3 h. Although sulfate removal was maintained at around 30% at HRT of 2 h, the free sulfide concentration increased to above 110 mg/L. Gaseous and dissolved sulfides usually cause physical–chemical and biological constraints in anaerobic digestion, which may lead to process failure. There have been many studies on sulfide inhibition in anaerobic digestion processes (Chen et al., 2008). The thresholds for digestion inhibition are in wide and confusing ranges of 150–1100 mg/L for dissolved sulfide and 50–250 mg/L for free sulfide (Omil et al., 1995). Paula and Foresti (2009) found that 100–500 mg/L dissolved sulfide showed no inhibition in anaerobic reactors. Stucki et al. (1993) considered that the anaerobic digestion was sensitive to undissociated H_2S above 50 mg/L when pure complete SRB was used in sulfate reduction. In the study of Kaksonen et al. (2004) on sulfide toxicity with batch kinetic experiments, they found that the inhibition concentration of dissolved sulfide for ethanol and acetate oxidation were 248 and 356 mg/L, respectively, while the corresponding values of free sulfide were 84 and 124 mg/L. Consequently, they concluded that ethanol oxidation was more readily inhibited by sulfide toxicity than acetate oxidation. In this UASB, there was no obvious digestion inhibition with dissolved sulfide stably below 300 mg/L. Although free sulfide increased sharply to 110–123 mg/L at HRT of 2 h, at other HRTs, it was kept in the range of 10–80 mg/L. Except at HRT of 2 h with OLR of 37.8 g/L d, there was no signal of digestion inhibition. Moreover, since no ethanol was detected in the effluent even at HRT of 2 h, acetate oxidation was more sensitive to sulfide toxicity than ethanol oxidation in this UASB. Despite the obvious decrease of COD removal at HRT of 2 h, sulfate removal was hardly affected. Therefore the free sulfide over 110 mg/L caused inhibition to methanogenesis, but had little influence on sulfidogenesis in this UASB reactor.

3.3. Gas production performance

The gas production rate was affected greatly by HRT and OLR variation (Fig. 2(g)). The average gas production rate was only 0.37 L/L d at HRT of 48 h. It increased to 5.92 L/L d at HRT of 3 h. At HRT of 2 h, owing to the acidification of the reactor, methanogenesis was inhibited greatly, resulting in a decline in the gas production to 4.05 L/L d. From Day 135, with HRT adjusted back to 6 h, the gas production rate stabilized in the range of 3.03–3.66 L/L d.

Fig. 2(h) shows gas composition variation during the operation. Except for the starting-up period and the operation with HRT of 2 h, methane percentage in the biogas kept mostly in the range of 70–80%. Accompanying the increase of VFA level in the reactor during HRT reduction, there was a descending trend of methane content in the biogas. With HRT above 6 h, the methane percentage was maintained around 80%. At HRTs of 4 and 3 h, the proportion of methane decreased to the range of 70–75%. At HRT of 2 h, owing to serious acidification in the reactor, this proportion decreased to only about 57%. However, the H_2S percentage in the biogas increased with HRT decrease. It increased from around 1.5% to 5.7% as HRT changed from 48 to 2 h.

According to the gas production and composition at all HRTs, the methane production rate (L/L d) and methane yield (L/gCOD) at different OLRs were obtained (Fig. 3(c)). It can be noticed that the methane production rate increased from 0.29 to 4.31 L/L d as the OLR changed from 1.4 to 24.7 gCOD/L d, then decreased to 2.31 L/L d at OLR of 37.8 gCOD/L d. However, there was little change in methane yield. The lowest value of 0.15 L/gCOD was noticed at OLR of 37.8 gCOD/L d with HRT of 2 h. At other OLRs, the methane yield kept in the range of 0.18–0.24 L/gCOD. At OLR of 12.3 gCOD/L d with HRT of 6 h, this value reached 0.23 L/gCOD. Although the methane yield in this study was lower than the theoretical methane yield of 0.35 LCH_4/gCOD, it was much higher than 0.069 L/gCOD in the study of Gimenez et al. (2011) with an anaerobic submerged

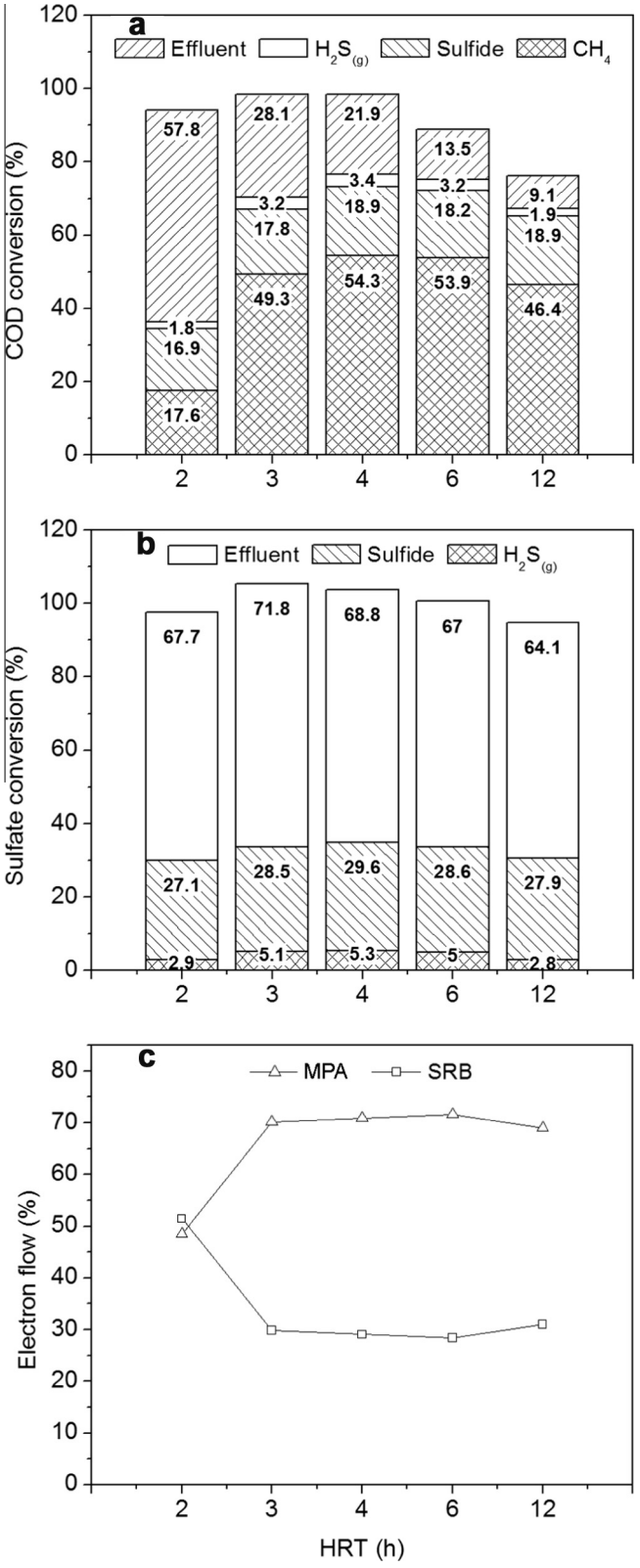

Fig. 4. COD, sulfate conversion and electron transmission at different hydraulic retention time (HRT): (a) COD conversion; (b) sulfate conversion; (c) electron flow variation.

membrane bioreactor treating sulfate-rich wastewater (with COD/S ratio of 2–8 and HRT of 6–21 h). In some other studies on sulfate-rich wastewater digestion (Damianovic and Foresti, 2007; Dinkel et al., 2010), no methane was detected in the biogas due to the predominance of sulfidogenesis. In this study, methane was always the main component in the biogas, which indicated methane production by MPA predominated in this UASB.

3.4. Variation of COD, sulfate conversion and electron transmission

According to CH_4 and H_2S composition in biogas, sulfide and residual COD in the effluent, COD conversion proportions at different HRT were obtained according to stoichiometry. Sulfate conversion was also analyzed with the contents of different forms of sulfur. The data in Fig. 4(a) indicate that the proportion of COD used for methane production was around 50% with HRT in the range of 3–12 h. When the HRT was reduced to 2 h, only 17.6% of influent COD was converted into methane, and 57.8% of COD was left in the effluent. However, the influence of HRT variation on COD utilization for sulfate reduction was not so obvious. At HRT of 2 h, there was still 18.7% of COD used for sulfate reduction to sulfide, which was very close to the proportions at other HRTs.

Fig. 4(b) shows there was little influence of HRT variation on the sulfate conversion. The proportion of sulfate converted into gas H_2S fluctuated in the range of 2.8–5.3%, whereas aqueous sulfide in the effluent accounted for 27.1–29.6% of influent sulfate. The sum of the proportions of gas H_2S and aqueous sulfide was 30–35% of total sulfate, which accorded well with the data of sulfate removal in Fig. 3(b).

According to methane, aqueous sulfide and gas H_2S production in the reactor, the percentages of electron utilized by MPA and SRB were calculated with the following equations (Hoa et al., 2007):

$$\text{Percentage of electron flow by MPA} = \text{CH}_4\text{-COD}/(\text{CH}_4\text{-COD} + \text{H}_2\text{S-COD}) \quad (1)$$

$$\text{Percentage of electron flow by SRB} = \text{H}_2\text{S-COD}/(\text{CH}_4\text{-COD} + \text{H}_2\text{S-COD}) \quad (2)$$

in which CH_4-COD = moles of CH_4 produced $\times$ 64 g, H_2S-COD = moles of sulfide produced in gas and water $\times$ 64 g.

As shown in Fig. 4(c), the percentage of electrons utilized by MPA was stable at around 70% with HRT above 3 h. However, this percentage decreased to only 48.5% at HRT of 2 h, which indicated that methane production was inhibited greatly. With HRT in the range of 3–12 h, SRB accounted for 28.4–31.0% of electrons utilization. At HRT of 2 h, while COD removal and methane production were refrained greatly, sulfate removal was hardly affected. Consequently, the percentage of electrons utilized by SRB increased to 51.5%.

It was reported that COD removal and electron flow by SRB always predominated over MPA in digestion with low COD/sulfate values (Omil et al., 1995). Dries et al. (1998) used an acetate-fed EGSB reactor to treat sulfate-rich wastewater, and found the proportion of electron flow to SRB kept above 80% after 28 days operation. Hoa et al. (2007) found that this percentage was in the range of 80–85% in a UASB system with a COD/sulfate ratio of 2. In the study of Li et al. (1996) with benzoate as the substrate, 87% of the electrons were utilized by SRB at COD/sulfate ratio of 0.75. After changing the substrate of a sulfate-reducing fluidized-bed reactor from lactate to ethanol, Kaksonen et al. (2003) increased the percentage of electrons utilized by SRB from 60–75% to 77–95%. However, in this UASB, the percentage of electrons utilized by SRB was much lower than that utilized by MPA except at HRT of 2 h. The electron flow characteristics indicated MPA predominated over SRB in organics removal and electrons utilization. Consequently, this UASB was more efficient in organics removal and methane production.

Table 1
Specific methanogenic activity (SMA) and specific sulfidogenic activity (SSA) of the granular sludge with different substrates.

Substrate	Without sulfate	With sulfate of 3000 mg/L	
	SMA (gCOD/gVSS/d)	SMA (gCOD/gVSS/d)	SSA (gCOD/gVSS/d)
$H_2 + CO_2$	0.192	0.095	0.464
Acetate	1.748	0.951	0
Ethanol	0.255	0.909	0.324
Wastewater	1.765	1.683	0.389

Table 2
Archaea composition of the granular sludge.

Phylogenetic group	No. of clones	Percentage (%)	Similarity (%)
Methanosaeta concilii GP6	36	69.2	99
Methanobacterium sp.	14	26.9	96
Desulfurococcus fermentans	2	3.9	85

3.5. Specific methanogenic activity and specific sulfidogenic activity of the granular sludge

The activity test results in Table 1 show that acetate was readily used for methane production. In the tests with acetate as substrate, the SMA without and with sulfate was 1.748 and 0.951 gCOD/gVSS d, respectively, which indicated that methane production was obviously refrained by high sulfate. The result that the SSA of acetate was zero indicated acetate could not be utilized as a carbon donor during sulfate reduction, and SRB in the granular sludge belonged to incomplete oxidizers. With 80% H_2 + 20% CO_2 as the substrate, the SSA of the sludge reached 0.464 gCOD/gVSS d, which was 2.4 and 4.9 times of SMA without and with sulfate, respectively. It was reported that hydrogenotrophic SRB always outcompeted hydrogenotrophic MPA at limited hydrogen circumstances (Chen et al., 2008). Consequently, in this UASB treating sulfate-rich wastewater, hydrogen was more readily utilized by SRB than by MPA. To ethanol, the SMA with sulfate was much higher than that without sulfate, and the SSA was even higher than the SMA without sulfate. These results indicated ethanol was an excellent carbon source for SRB in sulfate reduction. During digestion, the conversion of ethanol into acetate was greatly accelerated in the presence of sulfate (Lens et al., 1998), and the methane production velocity was also enhanced. It can also be noticed that the SMA utilizing acetate was very close to that utilizing ethanol in the presence of sulfate. Nevertheless, in the presence of high sulfate, the SMA utilizing the synthetic sulfate-rich wastewater was much higher than that with acetate or ethanol as the sole substrate. This indicated there was a mutual acceleration between ethanol and acetate biodegradation during high sulfate situation. With acetate as the sole substrate, methane production by MPA was refrained by high sulfate. With ethanol as the sole substrate, methane production was controlled by the acetate generation rate during acetogenesis and sulfidogenesis. In the digestion of the sulfate-rich wastewater containing both acetate and ethanol, SRB had a higher affinity for ethanol than for acetate (Vallero et al., 2005). When ethanol was utilized by SRB in sulfate reduction, more energy and acetate were supplied to MPA for methane production. Consequently, the granular sludge had a high SMA value with this sulfate-rich wastewater containing both ethanol and acetate.

With the results of activity test, the main anaerobic digestion processes for the wastewater containing ethanol and acetate can be expressed as follows: (1) acetogenesis, $C_2H_5OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$; (2) sulfidogenesis, $2C_2H_5OH + SO_4^{2-} \rightarrow 2CH_3COO^- + HS^- + H^+ + 2H_2O$, $4H_2 + H^+ + SO_4^{2-} \rightarrow HS^- + 4H_2O$; (3) methanogenesis, $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$.

3.6. Microorganism composition of 16S rDNA gene cloning

In the archaea of the granule sludge, there were only three kinds of phylogenetic groups (Table 2). Among the 52 clones of archaea, *Methanosaeta concilii GP6* and *Methanobacterium* sp. accounted for 36 (similarity 99%) and 14 (similarity 96%) clones, respectively. The other 2 clones were related to *Desulfurococcus fermentans*

Table 3
Bacteria composition of the granular sludge.

Phylum	Phylogenetic group	No. of clones	Percentage (%)	Similarity (%)
Firmicutes	*Clostridium sporogenes*	2	2.2	89
	Lactobacillus curvatus	1	1.1	78
	Ruminococcaceae bacterium	2	2.2	88
	Clostridium sp.	2	2.2	90
Proteobacteria	*Geobacter* sp.	16	17.5	96
	Desulfitobacterium dehalogenans	1	1.1	97
	Desulfovibrio fructosovorans	9	9.9	97
	Desulfovibrio sp.	4	4.4	98
	Geobacter uraniireducens	2	2.2	97
	Syntrophus aciditrophicus	1	1.1	92
	Syntrophobacter fumaroxidans	2	2.2	99
	Pseudomonas extremaustralis	4	4.4	99
	Acinetobacter sp.	2	2.2	99
	Pelobacter propionicus	1	1.1	98
	Campylobacter	1	1.1	99
Bacteroidetes	*Sphingobacterium* sp.	3	3.3	78
	Owenweeksia hongkongensis	1	1.1	89
	Dysgonomonas mossii	1	1.1	89
	Marinilabilia salmonicolor	2	2.2	87
	Alistipes putredinis	1	1.1	91
Spirochaetes	*Spirochaeta caldaria*	4	4.4	89
Chloroflexi	*Anaerolinea thermophila*	10	11.0	89
Synergistetes	*Aminomonas paucivorans*	13	14.3	92
Actinobacteria	*Rubrobacter xylanophilus*	1	1.1	84
Nitrospirae	*Thermodesulfovibrio yellowstonii*	3	3.3	88
Thermotogae	*Kosmotoga olearia*	1	1.1	89
Dictyoglomi	*Dictyoglomus turgidum*	1	1.1	80

(similarity 85%), which could use sulfur to generate energy and hydrogen during the fermentation of various carbohydrates (Perevalova et al., 2005). *Methanosaeta* plays an important role in removing organics from wastewater, and it can only use acetate as energy source to produce methane (de Lucena et al., 2011). In the influent of the UASB reactor, there was about 1000 mg/L acetate and 1000 mg/L ethanol. During the whole operation, no ethanol was detected in the effluent, whereas a quantity of acetate remained in the effluent. Thus the enough acetate in the reactor made *Methanosaeta* predominate in the granular sludge (Kobayashi et al., 2011), and the proportion of *Methanosaeta concilii GP6* in archaea reached 69.2%. *Methanobacterium* sp. can produce methane with H_2 and CO_2 (Luo and Angelidaki, 2012). As described in activity test with H_2 and CO_2 as substrate, SRB took predominance over MPA in H_2 utilization. Moreover, there was no H_2 detected in the biogas of the UASB. Consequently, *Methanobacterium* sp. did not use much H_2 to produce methane, and methane was mainly generated by *Methanosaeta concilii GP6* with acetate as substrate in this UASB reactor.

Data of bacteria cloning results in Table 3 show there were 10 phyla including 27 phylogenetic groups detected. Among the 27 groups, there were 3 groups of *Desulfovibrio*- like SRB (17 clones) and 1 group of *Desulfitobacterium dehalogenans* (1 clone). *Desulfitobacterium dehalogenans* was not seen as SRB because it could not reduce sulfate but sulfite, thiosulfate and sulfur into sulfide (Utkin et al., 1994). Both *Desulfovibrio fructosovorans* (9 clones) and *Desulfovibrio* sp. (4 clones) in *Proteobateria* could reduce sulfate with ethanol and H_2 as substrates. However, *Thermodesulfovibrio yellowstonii* (3 clones) in *Nitrospirae* could not utilize ethanol but H_2 in sulfate reduction (Sekiguchi et al., 2008). The total percentage of these SRB in this UASB with COD/sulfate ratio of 1 only amounted to 17.6%, which was lower than the value of 20.45% in an anaerobic CSTR (continuously stirred tank reactor) by Zhao et al. (2010) with ethanol and acetate as substrates and COD/sulfate of 2. *Desulfovibrio* are commonly referred to as incomplete oxidizing SRB (Kunapuli et al., 2010), and they cannot utilize acetate directly as an electron donor for sulfate reduction. Therefore, the SRB in the granular sludge mainly utilized ethanol as electron donor and converted it into acetate during sulfate reduction.

Among the other bacteria, *Clostridium* and *Syntrophobacter* are related to H_2 production with carbohydrates (Chang et al., 2008). Although there were also some other bacteria such as *Aminomonas*, *Anaerolinea* and *Spirochaeta*, they could not oxidize ethanol or acetate. They might work in endogenous decomposition to some polycarbohydrates. Consequently, according to the results of sludge activity test and microorganism composition, the main digestion pathways of ethanol and acetate in this UASB are illustrated in Fig. 5.

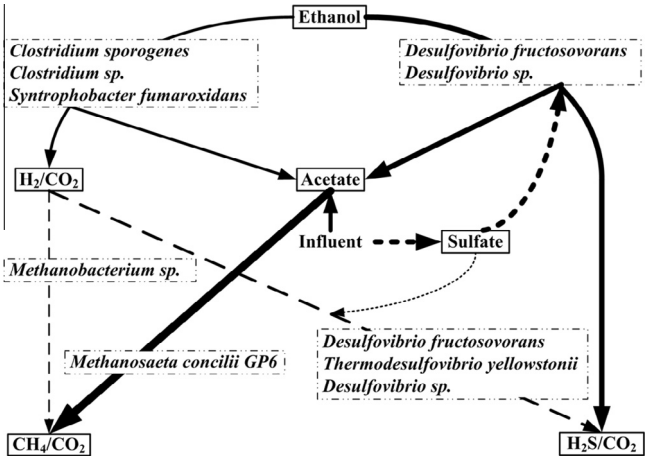

Fig. 5. Digestion pathways of ethanol, acetate and sulfate with functional microorganisms.

In this reactor, ethanol was mainly converted into acetate by *Desulfovibrio* in sulfate reduction. A small portion of ethanol was used by *Clostridium* and *Syntrophobacter* in H_2 production. Methane was mainly produced by *Methanosaeta concilii GP6* with acetate as substrate. Because *Desulfovibrio* outcompeted *Methanobacterium* sp. at limited hydrogen circumstances (Chen et al., 2008), most of H_2 generated during ethanol conversion was utilized by *Desulfovibrio* as substrate during sulfate reduction. However, because there was not much H_2 generation in this UASB, sulfate was mainly reduced by *Desulfovibrio* with ethanol as substrate.

4. Conclusion

In this UASB, COD removal and methane yield, respectively, maintained above 80% and 0.18 LCH_4/gCOD with HRT above 6 h and OLR below 12.3 g/L d. When HRT was further decreased, VFA accumulation resulted in COD removal deterioration, whereas sulfate removal kept around 30%. At HRT of 2 h, free sulfide increased above 110 mg/L and caused digestion inhibition. COD and electron flow were mainly utilized by MPA, and methane was generated by *Methanosaeta* with acetate as substrate. SRB accounted for 17.6% of the bacteria and belonged to incomplete oxidizers, which utilized ethanol rather than acetate in sulfate reduction.

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