JOURNAL OF ENVIRONMENTAL SCIENCES 135 (2024) 353–366

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JES JOURNAL OF ENVIRONMENTAL SCIENCES

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Research Article

The effect of slime accumulated in a long-term operating UASB using crude glycerol to treat S-rich wastewater

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ARTICLE INFO

Article history:
Received 26 September 2022
Revised 15 November 2022
Accepted 15 November 2022
Available online 24 November 2022

Keywords:
Sludge flotation
Granular sludge
Slime-like substances
Glycerol fermentation
Sulfate reduction
Removal efficiency

ABSTRACT

An up-flow anaerobic sludge blanket (UASB) reactor targeting sulfate reduction was operated under a constant TOC/S-SO_4^{2-} ratio of 1.5 ± 0.3 g C/g S for 639 days using crude glycerol as carbon source. A filamentous and fluffy flocculant material, namely slime-like substances (SLS), was gradually accumulated in the bioreactor after the cease of methanogenic activity. The accumulation of SLS was followed by a decrease in the removal efficiencies and a deterioration in the performance. Selected characteristics of SLS were investigated to explore the causes of its formation and the effect of SLS on the UASB performance. Results showed that glycerol fermentation and sulfate reduction processes taking place in the reactor were mainly accomplished in the bottom part of the UASB reactor, as the sludge concentration in the bottom was higher. The accumulation of SLS in the UASB reactor caused sludge flotation that further led to biomass washout, which decreased the sulfate and glycerol removal efficiencies. Batch activity tests performed with granular sludge (GS), slime-covered granular sludge (SCGS) and SLS showed that there was no difference between GS and SLS in the mechanism of glycerol fermentation and sulfate reduction. However, the specific sulfate reduction rate of GS was higher than that of SLS, while SLS showed a higher glycerol fermentation rate than that of GS. The different rates in GS and SLS were attributed to the higher relative abundances of fermentative microorganisms found in SLS and higher relative abundances of sulfate reducing bacteria (SRB) found in GS.

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https://doi.org/10.1016/j.jes.2022.11.011

Introduction

Despite anaerobic digestion is a well-known process in wastewater treatment to remove different types of organic pollutants, anaerobic biotechnology is also an alternative for sulfate-rich wastewater treatment. The success of the sulfate reduction process has been proven using a variety of carbon sources, such as methanol (Vallero et al., 2003), ethanol (Wu et al., 2018), butanol (Sarti and Zaiat, 2011), or crude glycerol (Fernández-Palacios et al., 2019). Following the sulfate reduction to sulfide, recovering valuable metals from metal-sulfides precipitation has been applied in full-scale installations by Paques company (Huisman et al., 2006). Further partial oxidation of sulfide to elemental sulfur as a value-added product to valorize S-rich effluents has also been reported recently (Mora et al., 2020a).

In order to further extend the application of valorizing sulfate-rich effluents, it is necessary to improve the robustness of biological processes by stabilizing the bioreactor, optimizing operating conditions and avoiding inhibitory compounds accumulation. Management strategies that are insufficiently evaluated may exacerbate risks, which further results in unexpected process failure and economic losses from their commercial application (Westerholm et al., 2018). Several problems have been identified in the long-term operation of UASB reactors. For example, fat and oil attached onto the biomass or the high up-flow velocity has been reported to lead to sludge flotation and the washout of biomass, thus decreasing the COD removal efficiency (RE) and leading to process failure (Jeganathan et al., 2006; Rizvi et al., 2015). Lu et al. (2015b) also described that the lack of consumption of excess extracellular polymeric substances produced and the biogas generated in an UASB reactor treating starch-rich wastewater resulted in the flotation of sludge that was not able to settle, which eventually led to sludge washout and the clogging of the outlet of the reactor. The required cleaning procedures due to clogged outlets or the reinoculation of biomass due to washout increase operating costs. Furthermore, when treating some specific wastewater (such as methanol-containing wastewater) in UASB reactors, the disintegration of GS increased the washout of sludge, which not only reduced the quality of the outlet and decreased the COD RE, but also increased the risk of failure (Lu et al., 2015a). Therefore, long-term evaluation of the stability performance of reactors for sulfate-rich effluents treatment is needed.

Many studies have already reported several factors affecting the anaerobic treatment of sulfate-rich wastewaters during the long-term operation, including the carbon source type, temperature, pH, sulfate loading rate and carbon to sulfur (C/S) ratio (Lopes et al., 2007; Mora et al., 2020b; Shin et al., 1996; Vallero et al., 2004). Anaerobic methanogenic sludge can be adapted to sulfidogenic conditions in order to achieve the treatment of sulfate-rich wastewaters (Lens et al., 2002). However, it can take a long time for SRB to outcompete methanogens. In order to adapt to the sulfate reduction conditions, previous studies showed some alternatives to speed up the start-up of the process such as adding a pure culture of SRB (Omil et al., 1997b) or using SRB enriched-biomass to target specific organic compounds (Kaksonen and Puhakka, 2007). In addition, an optimum COD/S-SO_4^{2-} ratio may also accelerate the adaptation period. Fernández-Palacios et al. (2019) investigated different sulfate and organic loading rates in a sulfidogenic UASB reactor. It took 30 days after the inoculation of the reactor to reach over 80% of sulfate RE at COD/S-SO_4^{2-} ratio of 3.8 g O_2/g S-SO_4^{2-}, while only 16 days after the inoculation, sulfate RE reached an 80% at COD/S-SO_4^{2-} ratio of 5.4 g O_2/g S-SO_4^{2-} and an organic loading rate of 15.8 kg O_2/m^3/day (Fernández-Palacios et al., 2021). Temperature shocks could also be a strategy to speed up the process of sulfidogenesis outcompetition over methanogenesis (Jung et al., 2019).

In addition, sludge granulation is an indispensable and critical factor for the robust treatment of wastewater. Granular biomass makes a significant contribution in maintaining the dynamic balance and the stability of reactors operation (Lu et al., 2015a). However, some studies found that non-granular particles were formed during sulfate reduction processes. For example, Vallero et al. (2003) reported the formation of thin fluffy flocculent particles in the treatment of synthetic wastewater containing methanol and sulfate in an UASB reactor inoculated with GS. Similarly, granules were disintegrated and filamentous and fluffy particles aggregated in an expanded GS bed reactor for sulfate reduction using methanol (Weijma et al., 2000). Weijma et al. (2000) reported negative effects of sulfate reduction on granulation. However, there is a lack of previous studies in the characterization of fluffy flocculent particles, their formation and potential effects on bioreactor performance as often they are not positive.

When acetate, cheese whey, pig slurry, crude glycerol and vinasses were selected as carbon sources for sulfate reduction process, crude glycerol showed better results and it was the most promising carbon source to reduce sulfate compared with others tested carbon sources (Mora et al., 2018). Crude glycerol is an inexpensive by-product mainly produced in the biodiesel production industry. Approximately, 0.1 kg of crude glycerol is produced per kg of biodiesel (Kumar et al., 2019). As a way to valorize this waste product from the biodiesel industry, crude glycerol has been applied in many processes such as the bioconversion of glycerol to biodiesel (Chen et al., 2018), methane production and hydrogen production in anaerobic digestion (Baba et al., 2013), composting (Fehmberger et al., 2020) and sulfate reduction (Mora et al., 2020a, 2020b). However, in a previous study (Fernández-Palacios et al., 2021), the long-term performance of an UASB to treat sulfate-rich wastewater using crude glycerol as a carbon source lead to the decrease of sulfate and organic compounds removal efficiencies, accompanied by the accumulation of a sticky flocculent SLS that surrounded the granular biomass. The present work aims at characterizing such SLS that accumulated in the UASB reactor, exploring the causes of its formation and the effect that this substance can have on the stability of the UASB performance over long-term periods. Batch activity experiments were set up to determine the difference between GS and SLS in the rates and the mechanism of sulfate reduction and organic compounds fermentation. SLS was characterized from a physical-chemical point of view including the analysis of microbial diversity, scanning electron microscopy (SEM), fourier transform infrared spectroscopy (FTIR) and fatty acid methyl esters (FAMEs).

1. Materials and methods

1.1. Experimental setup of the sulfidogenic UASB reactor

A 2.5 L UASB reactor was used for the treatment of sulfate-rich wastewater using crude glycerol as carbon source to produce sulfide. Six sampling ports located along the UASB reactor (at 7, 20, 30, 45, 54 and 72 cm from the bottom to the top of the UASB), named UASB1 to UASB6, respectively, were used to collect samples along the height of the reactor during its long-term operation. A schematic diagram of the process is presented in Appendix A Fig. S1. The UASB reactor was operated under constant conditions for 639 days, and the sulfate inlet concentration was set to 250 mg S-SO_4^{2-}/L, while the crude glycerol to sulfate ratio (TOC/sulfate) was steadily maintained at 1.5 ± 0.3 g C/g S. Detailed operating conditions of the UASB reactor are presented in Appendix A Table S1. GS to inoculate the reactor was obtained from a full-scale anaerobic UASB digester from a pulp and paper recycling industry (UIPSA, Spain). The initial volatile suspended solids (VSS) concentration of GS was 27.1 g VSS/L. However, the UASB reactor accumulated viscous, flocculent SLS along the operation, which are shown in Appendix A Fig. S2. UASB dimensions, equipment, mineral medium composition and crude glycerol characteristics are further detailed in Fernández-Palacios et al. (2021).

1.2. Assessment of stratification in the UASB reactor

The stratification of the UASB reactor was assessed by collecting samples from six sampling ports of the UASB reactor under two different situations: on day 198, when the reactor was properly operating under sulfidogenic conditions, and on day 575, when a large amount of SLS had already accumulated in the reactor and sulfate RE were dramatically reduced. The analysis of the inlet, outlet and the sampling ports of the reactor included monitoring of sulfate, thiosulfate, total dissolved sulfide (TDS), volatile fatty acids (VFAs), glycerol, total organic carbon (TOC), total inorganic carbon (TIC) and total carbon (TC). The calculating process of volumetric rates and mean cell residence time (MCRT) are presented in Appendix A supplementary methods. The sludge particle size was assessed at different bed heights of the UASB reactor (UASB1, UASB2 and UASB3) on days 50, 149 and 230, where the particle size distribution (PSD) and the median diameter of sludge particles (D(0.5)) were detected. D(0.5) was chosen, because the median is relatively unaffected by skewed distributions or extreme scores at end of distribution, compared to the mean (Field, 2013).

1.3. Batch tests

Since VFAs (mainly acetate and propionate) are reported as the most common intermediate products of glycerol degradation (Bertolino et al., 2014; Dinkel et al., 2010; Mora et al., 2020b; Zhou et al., 2022); acetate, propionate and glycerol were used as carbon sources to study the sulfate reduction process in batch tests. In order to study whether the decrease of the sulfate RE in the UASB reactor was due to the presence of SLS, sludge was collected from the UASB reactor on day 315 (when SLS covered GS) and on day 431 (when a large amount of SLS had already accumulated in the reactor), as shown in Appendix A Fig. S2. Table 1 summarizes the operating conditions of activity tests performed with different carbon sources.

The sludge collected from UASB1 on day 315 was separated into GS and slime-covered granular sludge (SCGS) (Appendix A Fig. S3). The SCGS was the sludge taken directly from the reactor without any treatment, which contained GS covered with SLS, whereas GS was granular sludge that had been cleaned to remove SLS. The cleaning process is presented in Appendix A Supplementary data. 150 mL of mineral medium were fed to both GS and SCGS, and acetate and propionate were added as carbon sources. The initial concentrations were set to maintain the same conditions as the TOC/S ratio used along the long-term UASB operation.

Due to the low concentration of GS at UASB1, where almost all the biomass was completely surrounded by SLS (Appendix A Fig. S2C) on day 431, the sludge collected by that time was from UASB6. The sludge collected from UASB6 on day 431 was divided into GS and SLS. The GS and SLS were fed with 150 mL of mineral medium and pure glycerol (99%, Panreac, Spain) as the carbon source.

Once the sludge, mineral medium and carbon sources were added to the serum bottles, the gas phase of the bottles was refilled with N_2. Then bottles were immediately covered with rubber stoppers and aluminum caps and incubated at 35 ± 1°C and 150 r/min in a constant temperature shaker (NB-T205, N-Biotek). All batch tests were carried out in duplicate.

1.4. Analytical methods

Sulfur compounds analyzed included sulfate, thiosulfate and TDS. TDS was analyzed off-line by a sulfide selective electrode (9616BNWP, Thermo Scientific, USA) connected to a benchtop meter (Symphony, VWR, USA). Before TDS measurements, samples were diluted one-to-two for UASB samples and one-to-twenty for activity tests samples with a sulfide antioxidant buffer (SAOB), which composition is described by Mora et al. (2020a). Sulfate and thiosulfate were analyzed by ion chromatography (ICS-2000 system, Dionex, USA) with a suppressed conductivity detector using an IonPac AS18-HC column (4 × 250 mm, Dionex, USA). Prior to the analysis of sulfate and thiosulfate, samples were bubbled with nitrogen to avoid chemical oxidation of sulfide and diluted to one fifth with ultrapure water.

Prior to the analysis of carbon compounds, samples were also bubbled with nitrogen. Volatile fatty acids (VFAs) and alcohols (ethanol, 1,3-propanediol, n-butanol, 2,3-butanediol) were measured by high-performance liquid chromatography (HPLC, Ultimate 3000, Dionex, USA) equipped with an ICSep ICE-CPREGEL 87H3 column (7.8 mm × 150 mm) and a variable wavelength detector at 210 nm with a 6 mmol/L H_2SO_4 mobile phase at a flow rate of 0.5 mL/min. Samples for VFAs and alcohols were not diluted.

Total organic carbon (TOC), total inorganic carbon (TIC) and total carbon (TC) were determined in a TOC analyzer (multi N/C 2100S, analytikjena, Germany) equipped with a furnace at the catalytic high-temperature of 850°C. Samples for TOC analysis were diluted to one third with ultrapure water.

Table 1 – Conditions of batch activity tests using glycerol and VFAs as carbon source.

Sampling day	Carbon Source	TOC (mg C/L)	Sulfate (mg S/L)	TOC/S (g C/g S)	Biomass (g VSS/L)
315	Acetate	326	248	1.31	0.28 ± 0.08[a] 0.48 ± 0.06[b]
315	Propionate	328	248	1.32	0.35 ± 0.05[a] 0.86 ± 0.11[b]
431	Glycerol	324	240	1.35	0.39 ± 0.13[a] 0.38 ± 0.03[c]

[a] Granular sludge
[b] Slime-covered granular sludge
[c] Slime-like substance

The particle size of sludge was evaluated by a laser diffraction testing instrument (Mastersizer 2000, Malvern Panalytical, USA). The sludge samples were taken from different heights of the UASB reactor and measured in triplicates.

VSS were analyzed according to Standard Methods (APHA, 2012) to calculate UASB biomass concentration and the specific rates obtained during the batch tests.

1.5. *16S rRNA gene amplification for GS and SLS*

Identification of microbial populations was performed using Illumina platform on different samples. Sludge was collected from UASB1 and UASB6 on day 538 of the operation. SCGS samples were firstly centrifuged at 4000 r/min for 10 min and, the supernatant was discarded. GS and SLS were separated and manually transferred to a new falcon tube where distilled water was added. The centrifugation process was repeated 2 to 4 times until GS and SLS were separated in the falcon tubes. Then, GS and SLS were separated by plastic pipettes. After separation, samples were cleaned with 1XPBS (7.2 mmol/L $Na_2HPO_4{\cdot}12H_2O$, 2.8 mmol/L $NaH_2PO_4{\cdot}2H_2O$ and 0.13 mmol/L NaCl), and centrifuged at 14000 r/min during 5 min (3 times). PowerSoil™ DNA isolation kit (MoBio Laboratories, USA) was used to extract the genomic DNA from both samples. The quantity and quality of the extracted DNA were assessed by using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) and finally, DNA samples were preserved at -20°C for further analysis. Illumina MiSeq platform was used to performed amplicon sequencing 16S rRNA genes of both samples by the "Genomic and Bioinformatics service" of Universitat Autònoma de Barcelona amplifying the V3-V4 hyper variable region with the universal primers by Illumina (5'-CCTACGGGNGGCWGCAG-3') and (5'-GACTACHVGGGTATCTAATCC-3') selected from Klindworth et al. (2013).

The analytical methods of SEM, FT-IR, and FAMEs are shown in Appendix A Supplementary data.

2. Results

2.1. *Stratification and long-term performance of the UASB reactor*

The UASB reactor was operated under a constant sulfate loading rate of 5.0 ± 0.6 kg S/m^3/day and an organic loading rate of 7.3 ± 1.6 kg C/m^3/day for 639 days. The gas production rate in the UASB and the sulfate, TOC and glycerol RE are provided in Fig. 1 to illustrate the UASB performance. Further, more complete details of the experimental performance results of the UASB reactor can be found elsewhere (Fernández-Palacios et al., 2021). In brief, the operation was divided into four periods: stage I, from day 0 to day 16, corresponding to the start-up and GS adaptation period; stage II, from day 16 to day 100, corresponding to a period with a stable sulfate reduction with methane production; stage III, from day 100 to day 280, under stable sulfate reduction without methane production; and stage IV, from day 280 to day 639, when a decline in the sulfate RE and glycerol RE occurred. After the adaptation period, stable sulfate RE higher than 83% were achieved on stage II and stage III and it decreased to 38.8% on stage IV. In terms of carbon, over 97% of glycerol RE was observed on stage I, II and III, but it decreased to 64% on stage IV. It can be observed that the TOC RE was positively correlated with the gas production. When the gas production rate was higher than 60 mL/hr, the TOC RE was higher than 86% on stage I and II. A progressive decrease in the gas production was accompanied by the decrease of TOC RE on stage II and III. The pH in the outlet of the UASB reactor was maintained at 7.0-7.3 (Appendix A Fig. S4).

During stages II and III, a progressive degranulation of the sludge was observed when methanogenic activity stopped. Sludge particle size distribution over the operation period is shown in Appendix A Fig. S5. The particle size of the inoculated sludge below 200 µm accounted for 74% of the inoculated sludge. Compared with inoculated sludge, the proportion of 0-200 µm sludge in UASB1, UASB2 and UASB3 decreased on day 50 and day 149, accounting for 49.6% ± 4.5% in average. However, the proportion of particles smaller than 200 µm in UASB1, UASB2 and UASB3 increased to 70.6% ± 3.2% on day 230. Also, the proportion of sludge greater than 1000 µm increased from 2.1% in the inoculum sludge to over 7.3% in UASB1, UASB2 and UASB3 on days 50 and 149. However, on day 230, the proportion of particles larger than 1000 µm were reduced to 1.6% (in UASB2) and 1.7% (in UASB3), respectively. Results of D(0.5) revealed that granular size increased from day 50 to day 149 as the inoculum sludge D(0.5) was 105 µm, while the sludge D(0.5) in UASB1, UASB2 and UASB3 were higher than 150 µm on days 50 and 149 (Appendix A Fig. S6). However, the D(0.5) of the sludge measured at different heights of the UASB reactor decreased on day 230 of the operation. Results of D(0.5)

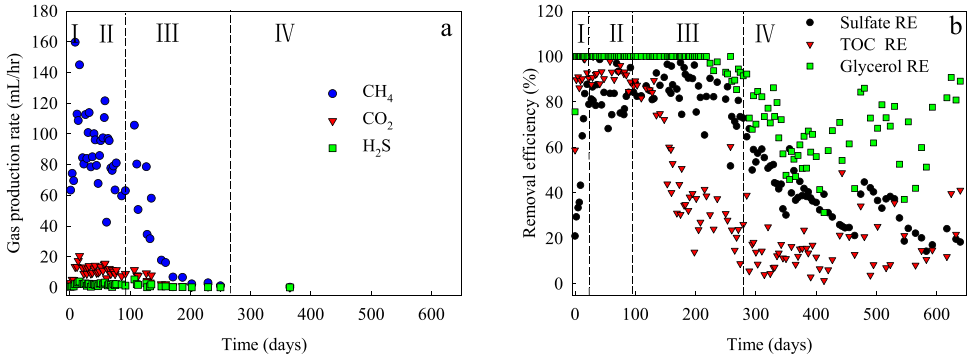

Fig. 1 – Gas production rate (a) and removal efficiency (b) of the sulfidogenic UASB reactor along 639 days.

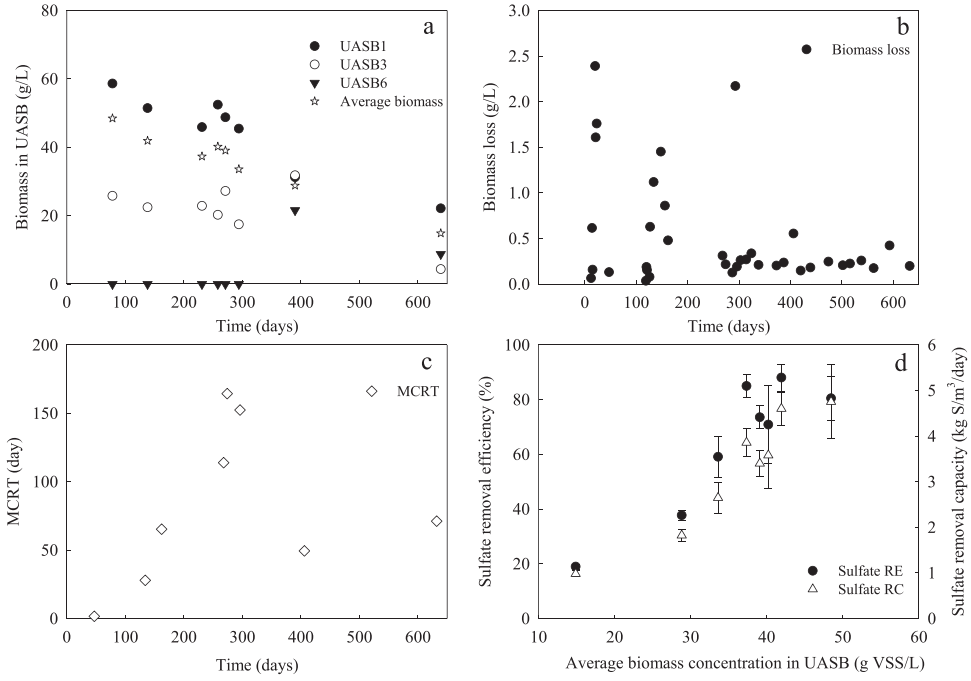

Fig. 2 – Variation of biomass in the UASB reactor (a), biomass loss (b), mean cell residence time (c) and biomass concentration in UASB versus sulfate removal efficiency and sulfate removal capacity (d).

and particle size distribution revealed that the granule size of the sludge increased on days 50 and 149 in UASB1, UASB2 and UASB3, but decreased on day 230 when no biogas was produced.

When the reactor was operated under sulfidogenic conditions with low biogas production on stage III, flocculent SLS gradually surrounded and covered GS (Appendix A Fig. S2b), later forming aggregates and attaching to the reactor wall (Appendix A Fig. S2c, S2d). Compared with GS, SLS was viscous and fluffy, which caused severe sludge flotation and biomass washout. As can be observed in Appendix A Fig. S2b, part of the sludge floated to the gas-liquid-solid separator and remained suspended on the top of the reactor after 316 days of operation and later on (Appendix A Fig. S2c, S2d).

Biomass concentration in the UASB reactor and washout of biomass are shown in Fig. 2. As shown in Fig. 2b, the biomass concentration in UASB1 showed a decline from 58.6 g/L on day 78 to 22.1 g/L on day 639. The biomass concentration in UASB3 was between 20 and 30 g/L before day 400 and dropped to 4.3 g/L on day 639. The biomass concentration in UASB6 was 0 g/L before day 300. Due to flotation, part of the sludge was retained at UASB6, where biomass concentration was 21.6 g/L on day 390 and dropped to 8.7 g/L on day 639. The average biomass concentration in the UASB reactor gradually dropped from 48.5 g/L to 14.9 g/L during 639 days operation. The TSS concentration in the UASB reactor showed the same trend as VSS (data not shown). The VSS/TSS ratio ranged from 74% to 87% during the whole operation, which shows that there was almost no accumulation of non-volatile suspended solids in the UASB reactor indicating that the main component of SLS accumulated in the reactor were volatile solids.

Fig. 2b shows the washout of biomass from the reactor. The biomass loss fluctuated in the range of 0.04 to 2.4 g/L before day 200. High sludge concentration losses were caused by an excess of biomass provided to the reactor in the inoculation stage coupled to the movement and raising of granules

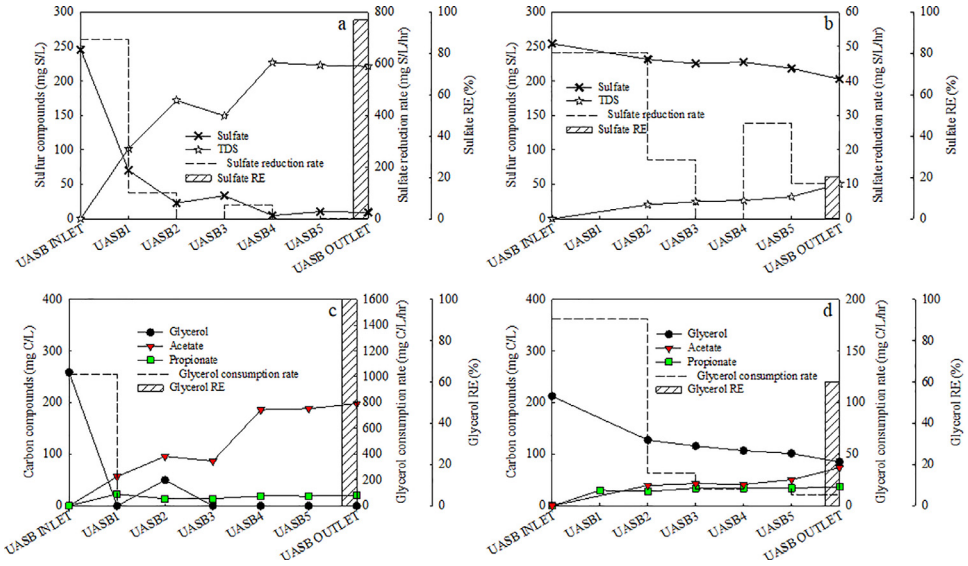

Fig. 3 – UASB sulfur and carbon concentration profile on day 198 (a, c) and on day 575 (b, d) of the long-term operation using crude glycerol as carbon source. a and b show sulfur species. c and d show carbon species. TDS represents total dissolved sulfide.

caused by methane bubbles produced, thus resulting in sludge washout. When there was no methane production in the reactor, the bed become a static bed, leading to a reduced sludge loss that remained stable between 0.1 to 0.6 g/L after day 260. The MCRT was below 65 day during the period of methane production while the sludge remained longer in the reactor during the period of non-methane production (days 200 to 300), as shown in Fig. 2c. The MCRT was maintained at 50 day after 400 days of operation. Fig. 2d shows that the biomass in the reactor correlated with sulfate RE (%) and sulfate removal capacity (kg S/m^3/day).

Stratification of the UASB reactor was investigated under sulfidogenic conditions. Fig. 3 shows the profiles of sulfur and carbon species on days 198, when the reactor was properly operating under sulfidogenic conditions, and on day 575, when UASB performance was deficient. The sulfate RE on day 198 was 96.2% (Fig. 3a) and decreased to 20.2% on day 575 (Fig. 3b), while the glycerol RE was 100% on day 198 (Fig. 3c) and decreased as well to 60% on day 575 (Fig. 3d). Fig. 3 also shows the rates calculated with the data obtained in the UASB stratification sampling events. The glycerol consumption rate and sulfate reduction rate from UASB inlet to UASB1 were the highest, compared with the rest of the sampling points of the UASB reactor. On day 198, sulfate reduction and glycerol degradation were mainly completed at the bottom part of the UASB reactor (UASB inlet to UASB2). However, sulfate reduction and glycerol degradation occurred along the whole UASB reactor on day 575 as the local activity of biomass was reduced with respect to the period of proper performance.

2.2. *Anaerobic activity tests of GS, SCGS, and SLS*

2.2.1. *GS and SCGS tests feeding acetate and propionate*

Acetate and propionate, often reported as main products of glycerol degradation, were selected as electron donors for preliminary sulfate reduction tests. In order to study the properties of SLS that accumulated in the reactor, GS and SCGS collected from UASB1 on day 315 were investigated by batch activity tests using acetate as the carbon source to assess the influence of SLS on the mechanism of sulfate reduction (Fig. 4). Fig. 4 shows that there were no changes in the acetate and sulfate concentrations in batch tests performed with GS and SCGS, which further confirmed that after 315 days of operation, acetoclastic methanogens and acetotrophic SRB were not present in the UASB reactor.

Fig. 5 shows the time course of sulfate reduction with GS and SCGS using propionate as carbon source. GS and SCGS both reduced sulfate to sulfide, while acetate and inorganic carbon were produced. The specific rates of propionate consumption and sulfate reduction of GS and SCGS are shown in Table 2. The propionate consumption rate and sulfate reduction rate of GS were 68% and 66% higher than those of SCGS, respectively.

2.2.2. *GS and SLS tests feeding glycerol*

In order to analyze the difference between GS and SLS and to assess the influence of SLS on glycerol fermentation, sludge withdrawn from UASB6 on day 431 was handled. Fig. 6 shows the activity test using glycerol as the carbon source. GS reduced sulfate throughout the test (Fig. 6a). Instead, SLS required an adaptation period as it started to reduce sulfate only after 24 hr (Fig. 6b). Glycerol was fermented both by GS and SLS leading to ethanol, 1,3-propanediol, formate, acetate and propionate production in both cases followed by ethanol, 1,3-propanediol, formate and propionate consumption accompanied by sulfate reduction and coupled to acetate and inorganic carbon accumulation throughout the test. However, the rates of accumulation and depletion of each compound in the tests with GS and SLS were different. Essentially, a much faster fermentation rate was observed for SLS with respect to GS, while a faster use of electron donors was observed for GS than for SLS. Table 3 shows the specific rates calculated for glyc-

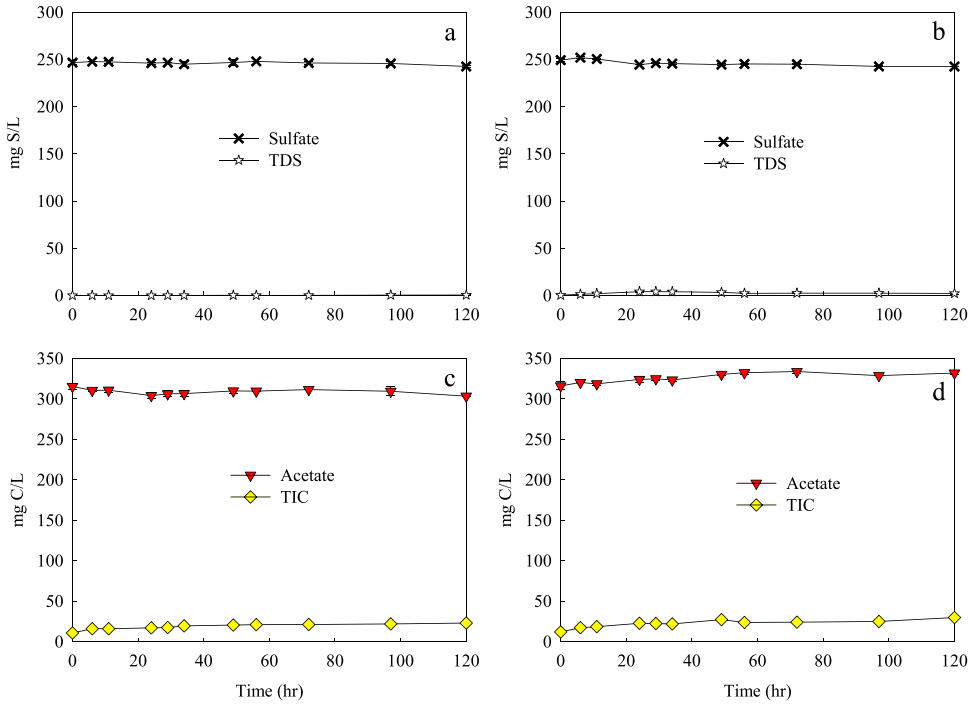

Fig. 4 – Activity tests of sludge obtained from UASB1 on day 315 feeding acetate and sulfate using GS (a, c) and SCGS (b, d). (a) and (b) show sulfur species, while (c) and (d) show carbon species where TDS and TIC correspond to total dissolved sulphide and total inorganic carbon, respectively.

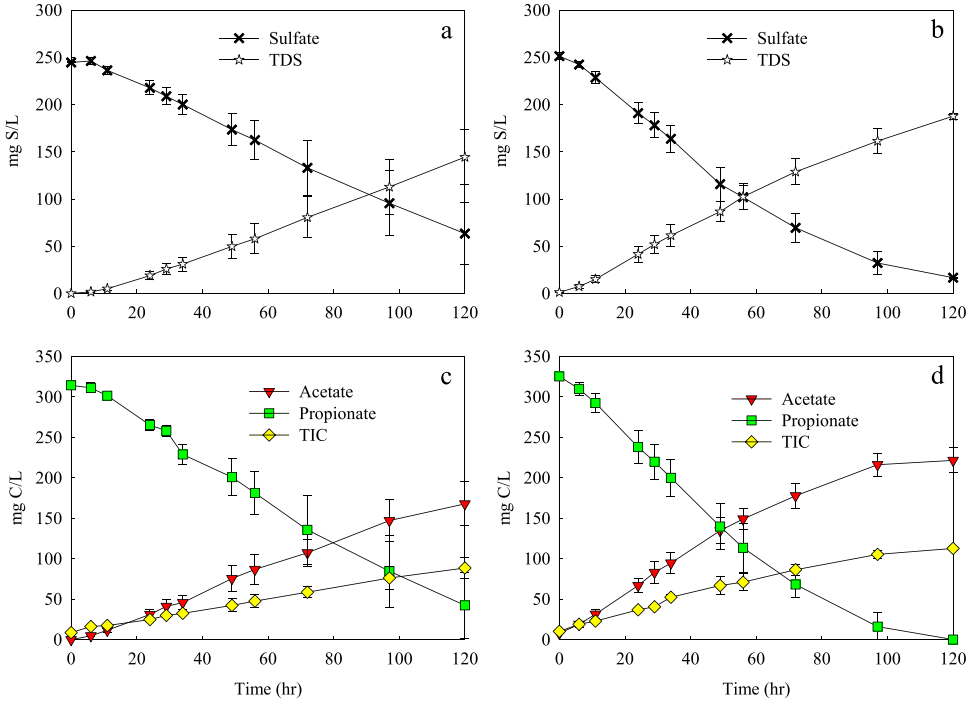

Fig. 5 – Activity tests of sludge obtained from UASB1 on day 315 feeding propionate and sulfate using GS (a, c) and SCGS (b, d). (a) and (b) show sulfur species, (c) and (d) show carbon species. TDS represents total dissolved sulfide. TIC represents total inorganic carbon.

Table 2 – Specific rates calculated for the sludge collected from UASB1 on day 315 feeding propionate and sulfate.

Sample	Time (hr)	Propionate consumption rate (mg C/hr/g VSS)	Sulfate reduction rate (mg S/hr/g VSS)
GS	6-120	6.9 ± 1.3	4.8 ± 0.6
SCGS	6-97	4.1 ± 0.8	2.9 ± 0.7

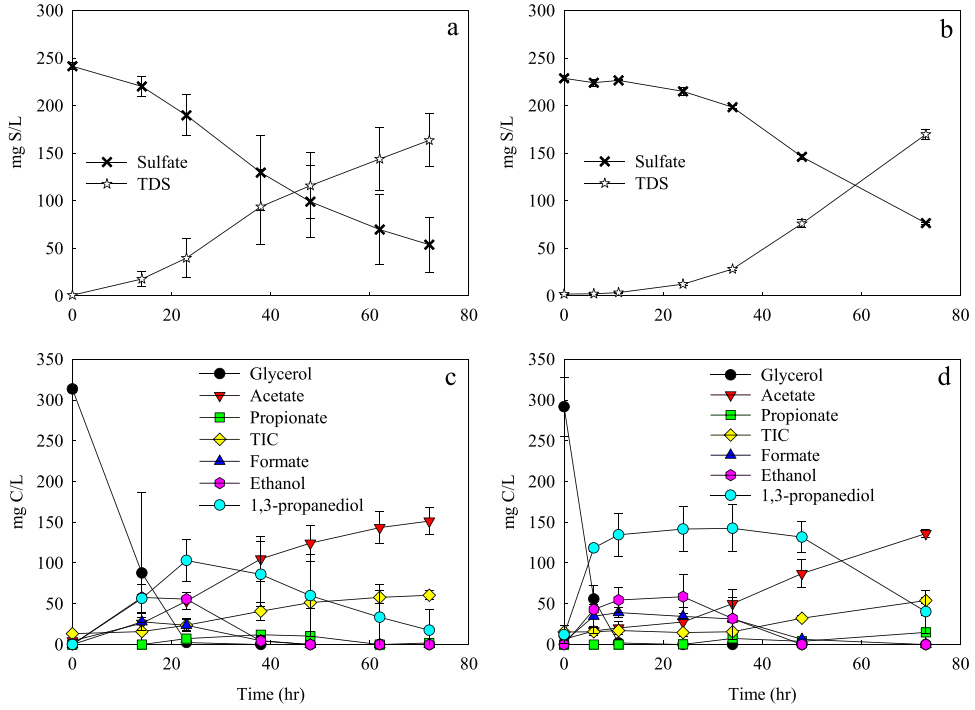

Fig. 6 – Activity tests of sludge obtained from UASB6 on day 431 feeding glycerol and sulfate using GS (a, c) and SLS (b, d). (a) and (b) show sulfur species, (c) and (d) show carbon species. TDS represents total dissolved sulfide. TIC represents total inorganic carbon.

Table 3 – Specific rates calculated for the sludge collected from UASB6 on day 431 feeding glycerol and sulfate.

Sample	Time (hr)	Glycerol consumption rate (mg C/hr/g VSS)	Sulfate reduction rate (mg S/hr/g VSS)
GS	0-23	33.1 ± 12.2	6.3 ± 3.4
	23-62	0	9.1 ± 1.7
SLS	0-11	70.1 ± 8.0	0.5 ± 0.5
	24-73	0	7.3 ± 2.7

erol consumption and sulfate reduction using GS and SLS in the tests. During the degradation of glycerol, the glycerol consumption rate of SLS was 2.1 times that of GS, and the sulfate reduction rate of GS was 1.2 times higher than that of SLS when the carbon source required for sulfate reduction was sufficient.

2.3. *Biological and physical-chemical characterization of GS and SLS*

The differences in microbial communities of GS and SLS were also studied. Fig. 7 compares the relative abundances (%) of the most abundant genus detected in the samples of GS and SLS on day 538 of operation using Illumina 16S rRNA sequencing. *Propionispora* and *Dysgonomonas* were the most abundant genus detected on day 358 in both samples. The relative abundance of genus *Propionispora* increased from 15.2% in GS to 21.5% in SLS, respectively. On the other hand, *Dysgonomonas* was most abundant in GS with a relative abundance of 13.2%, whereas the relative abundance of this genus was 9.9% in SLS. *Enterobacter, Klebsiella*, and *Helicobacter* genera raised their relative abundances in SLS if compared to GS, from 2.2% to 8.3%; from 2.1% to 8.5% and lastly from 0.3% to 6.4% respectively. *Desulfobulbus* decreased its relative abundance from 11.6% in

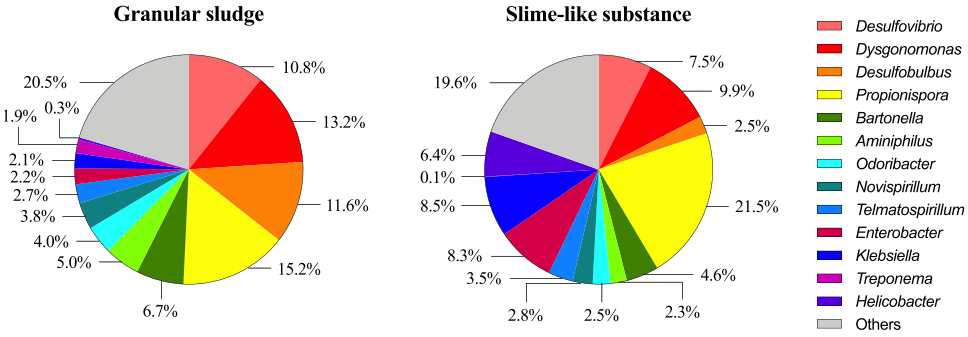

Fig. 7 – Relative abundance of genus detected in granular sludge (GS) and slime-like substance (SLS) on day 538.

GS to 2.5% in SLS, while *Desulfovibrio* decreased from 10.8% in GS to 7.5% in SLS.

Samples of SLS were collected for SEM analysis (Appendix A Fig. S7). Biofilm growth and a wide variety of microorganisms were observed in these samples. In some images, many fiber-like strings that are much larger than microbes can be also seen around the microbes.

The FTIR spectra obtained from the lyophilized SLS and GS is presented in Appendix A Figs. S8 and S9. Appendix A Fig. S8a shows the region from 4000 to 3000 cm^{-1}, where O-H stretching can be seen. Absorbance at wavenumbers below 3000 cm^{-1} shows C-H groups that are sp^3 hybridized, such as saturated fats or CH_3 groups (Appendix A Fig. S8b). Different bands can be seen between 1700-1600 cm^{-1} (Appendix A Fig. S8c) that indicate different secondary protein structures (α-helix, β-sheet, β-turn and random coil), which named the amide I bands. Amide II bands are shown in Appendix A Fig. S8d, which is indicative of proteins. The region from 1200 to 1100 cm^{-1} shows the indicative of C-O-C binding, typically as sugars bonds (Appendix A Fig. S9). The absorbance at 1150 cm^{-1} was 0.16 and 0.07 for SLS and GS, respectively.

The same UASB reactor was operated under different sulfate and organic loading rates in previous works (Fernández-Palacios et al., 2019; Fernández-Palacios, 2020). After the long-term operation varying COD/S-SO_4^{2-} ratios, SLS was also observed. Results of FAMEs analysis of SLS samples extracted from that operation after 490 days are presented in Appendix A Table S2. Palmitic acid was the main LCFAs identified and quantified compound in SLS from samples collected at different heights of the UASB reactor. Other interesting compounds that were detected in the samples but were not quantified were methyl esters and wax esters.

3. Discussion

A progressive decrease in methane production was accompanied by the decrease of TOC RE at a TOC/S-SO_4^{2-} ratio of 1.5 ± 0.3 g C/g S when crude glycerol was fed in the UASB reactor. At the same time, VFAs (mainly acetate, propionate) progressively accumulated on stage III and IV (Appendix A Fig. S4). However, the concentration of the non-ionic forms of VFAs (acetic acid and propionic mainly) were residual under the pH of operation considering the pKa of acetic and propionic acids, 4.75 and 4.87, respectively. The microbial diversity dynamics during the long-term operation of the UASB reactor was studied by Fernández-Palacios et al. (2021). The relative abundance of methanogens, including *Methanosaeta, Methanobacteria, Methanomicrobiales*, showed a downward trend. Methanogens were hardly detected after 230 days operation, which confirmed the washout of methanogens. In contrast, a remarkable increase in the relative abundance of SRB was observed. Illumina sequencing results were consistent with the results of methane production and VFAs accumulation, proving that methanogenic GS progressively switched to sulfidogenic sludge performing high sulfate RE.

The pH and sulfide concentration play a major role in the competition between SRB and methanogens (O'Flaherty et al., 1998). The growth rate of methanogens was higher than that of SRB at pH < 7.0, and opposite results were found at pH > 7.5. The pH in the inlet of the UASB reactor was over 7.5 during this study (Appendix A Fig. S4). The toxicity of sulfide is caused by undissociated sulfide molecules that permeate the cell membrane (Kaksonen and Puhakka, 2007). The inhibitory effect of H_2S on methanogens was greater than that on SRB (Sarti and Zaiat, 2011). Methane production by methanogenic archaea can be limited also by LCFAs, specifically palmitic acid (Deaver et al., 2020; Silva et al., 2016). Palmitic acid was identified as the main component among the LCFAs in the UASB reactor (Appendix A Table S2). Palmitic acid has a toxic effect on methanogenic archaea (Silva et al., 2016), and the adsorption of LCFAs onto microbial biomass can lead to mass transfer limitation of substrates (Pereira et al., 2005), which might be a potential factor for the inhibition of methanogenic activity.

The overall glycerol RE of the UASB reactor was over 90% with high sulfate RE (over 83%) before day 280. However, both the overall glycerol RE and sulfate RE started to decline after 280 days of operation. The decline of granules size was observed after the cease of methanogenic activity. Wu et al. (2016) found that granule size positively correlated with biogas production rate. Acetotrophic methanogens played a key role in granulation (Hulshoff Pol et al., 2004), and the lack of methane production resulted in poor sludge granulation (Mora et al., 2020b). Previous studies found that granules strength was reduced upon sulfidogenic operation of anaerobic reactors and the diameter of GS decreased (Kobayashi et al., 2015; Omil et al., 1997a), which might ex-

plain the observation of degranulation when methanogenic activity ceased. Weijma et al. (2000) found the disintegration of sludge in an expanded GS bed reactor treating methanol during the period of low biogas production, and reported that sludge particles were gradually covered by fluffy cotton-like material. Similarly, in the present study, when no biogas was produced in the UASB reactor, decrease in granuleś size was also observed followed by SLS accumulation.

As it can be seen in SEM images, the SLS presented a white cotton-like aspect. The formation of SLS may be attributed to the accumulation of intermediate metabolites in extracellular polymeric substances, such as fats, oils, greases, or polysaccharides (Jeganathan et al., 2006; Lu et al., 2015b). The formation of SLS may also be due to the continuous accumulation of other organic carbon substances in crude glycerol that were not completely degraded by GS, thus encircling the GS. It has been previously reported that crude glycerol contains FAMEs, free fatty acids, glycerides and LCFAs (Hu et al., 2012; Viana et al., 2012). LCFAs can form a layer on the surface of biomass and prevent the substrate from entering the biomass, thus leading to limited substrate diffusion into the granules (Viana et al., 2012). In addition, LCFAs can attach the biomass and cause its flotation (Viana et al., 2012). Hwu et al. (1998) found that treatment of LCFAs-containing wastewater in an UASB lead to methanogenesis inhibition and granular sludge flotation. LCFAs accumulated in the UASB reactor after long-term operation under sulfidogenic conditions in our work. Sludge flotation is a limiting factor for anaerobic treatment of wastewater with higher loading rates because this phenomenon can lead to a severe biomass washout and system performance deterioration (Jeganathan et al., 2006). As can be observed in Fig. 2d, the decrease in biomass concentration is accompanied by a decrease in sulfate RE and sulfate removal capacity, which indicated that sludge flotation lead to the washout of biomass and the drop of the reactor performance. This may explain the gradual decline of sulfate RE and glycerol RE at stage IV of the long-term UASB operation.

The evaluation of UASB reactor stratification can help to understand the effect of the sludge at different heights on sulfate reduction and degradation of organic compounds. It is observed that sulfate reduction and glycerol degradation were mainly completed at the bottom of the UASB reactor, which was due to the high biomass concentration at this height of the UASB reactor (UASB inlet to UASB2). The biomass at the bottom of the sludge bed (UASB inlet to UASB2) accounted for over 70% of the overall VSS of the reactor before 300 days of UASB operation. The biomass from UASB inlet to UASB2 also accounted for 57% and 78% on day 390 and day 639, respectively. Results are consistent with previous studies, which found that the higher methanogenic activity was observed at the bottom of the sludge bed in an UASB-anaerobic membrane bioreactor treating municipal wastewater because the concentration of VSS increased along with the decreasing height of the sludge bed (Mahmoud et al., 2004; Ozgun et al., 2019). Similarly to what was observed by Ozgun et al. (2019), the highest specific methanogenic activity was observed at the bottom part of the sludge bed, as the more active biomass remained near the inlet of the reactor, while the inactive biomass moved towards the top of the sludge bed or suspended on the top of the reactor.

GS, SCGS and SLS were also investigated by batch activity tests to identify the influence of SLS on the mechanism of sulfate reduction and glycerol degradation. When propionate was used as carbon source to reduce sulfate, SRB converted propionate to acetate and inorganic carbon. The mechanism of sulfate reduction with propionate observed herein is consistent with previous studies (Liamleam and Annachhatre, 2007; Muyzer and Stams, 2008). Since the same products were observed in the activity test performed with GS and SCGS using propionate to reduce sulfate as shown in Fig. 5, it can be concluded that the SLS presented the same mechanism of sulfate reduction. However, the specific sulfate reduction rate was higher in the case of GS than in that of SCGS, indicating that SLS may affect the mass transfer of the substrates, resulting in a decrease of the specific sulfate reduction rate.

In the activity tests performed with GS and SLS with glycerol as the carbon source to reduce sulfate, both GS and SLS fermented glycerol into formate, acetate, propionate, ethanol, 1,3-propanediol and inorganic carbon. In addition, the intermediate products of glycerol were consumed with sulfate reduction observed. This indicates that SLS is also capable of fermenting glycerol and reducing sulfate. When comparing the specific rates of glycerol fermentation between GS and SLS, SLS was more efficient in degrading glycerol than GS. The specific substrate utilization activity of GS and flocculant sludge was compared in UASB reactors processing wastewater from food industries (Oleszkiewicz and Romanek, 1989). They found that the flocculant sludge formed during the operation of an UASB reactor had a higher specific activity for the degradation of complex polymers than the GS in anaerobic processes, which is similar to the results found herein.

Fig. 6a and b show that sulfate reduction was not related to glycerol fermentation. Sulfate was reduced accompanied by the degradation intermediate products of glycerol degradation. This confirmed that the carbon sources used for sulfate reduction were formate, ethanol and 1,3-propanediol, in which the mechanism of sulfate reduction using glycerol as the carbon source is consistent with our previous work (Zhou et al., 2022). When comparing the specific rates of sulfate reduction between GS and SLS, SLS was found to be less efficient in reducing sulfate than GS. In addition, as can be seen in Fig. 6d, even if there was sufficient carbon source (formate, ethanol and 1,3-propanediol) to reduce sulfate, SLS still needed time to adapt and perform the sulfate reduction process, which may be caused by mass transfer limitation of sulfate. Compared with GS in the UASB reactor, GS covered by SLS may cause sulfate to be discharged from the UASB reactor before entering the cell due to mass transfer limitation, which may lead to a decrease in the sulfate reduction efficiency. Therefore, a significant reason for the reduced sulfate RE in the UASB reactor may also be that the SLS limited the mass transfer of sulfate, in addition to biomass washout from the UASB reactor.

The difference of specific rates between GS and SLS were also caused by the bacteria community differences. A wide range of microorganisms have been observed to ferment glycerol, including *Dysgonomonas* (Moscoviz et al., 2018), *Klebsiella* (Cheng et al., 2007), *Propionibacterium* (Himmi et al., 2000) *and Propionispora* (Abou-Zeid et al., 2004). Obligate anaerobic *Dys-*

gonomonas genus is able to convert glycerol to 1,3-propanediol. *Propionibacterium* and *Propionispora* were reported to convert crude glycerol to propionate and acetate (Abou-Zeid et al., 2004; Biebl et al., 2000; Himmi et al., 2000). Anaerobic *Propionispora* genus typically degrades organic carbon compounds in acidogenesis rather than acetogenesis. *Enterobacter* and *Klebsiella*, facultative anaerobes, are able to produce hydrogen and acetate through the fermentation of organic substrates (Hung et al., 2011; Joubert and Britz, 1987). *Helicobacter* is considered to be a microaerophile capable of oxidizing a variety of organic acids, such as formate, lactate, succinate and pyruvate (Joubert and Britz, 1987). In this work, the relative abundances of fermentative microorganisms in GS (33.0%) were lower than that in SLS (54.6%), which agrees with the lower glycerol fermentation rate in GS and higher fermentation rate in SLS.

Desulfovibrio is a genus of SRB reported to oxidize organic carbon compounds (including lactate, ethanol, malate, pyruvate, succinate) to acetate for sulfate reduction (Odom and Peck, 1981; Qatibi et al., 1991; Wu et al., 2018). *Desulfobulbus* genus can oxidize pyruvate, propionate and ethanol to acetate (Bak and Pfennig, 1991; Bertolino et al., 2012; Muyzer and Stams, 2008; Zeng et al., 2019). The main sulfate-reducer genera found herein were *Desulfobulbus* and *Desulfovibrio*, both reported as incomplete oxidizing SRB, which explains the accumulation of acetate in the batch activity tests. Compared to SLS, higher relative abundances of *Desulfobulbus* and *Desulfovibrio* in GS agree with the higher sulfate reduction rate found in GS, meaning that the populations of SRB were probably mainly growing in the core of the granules, what can also explain the decrease of sulfate RE when granules turned into slime-covered granules.

Batch activity tests and 16s rRNA gene analysis of the microbial communities revealed that GS played a more important role in sulfate reduction than SLS since GS contained more sulfate reducers (mainly *Desulfobulbus* and *Desulfovibrio*). In terms of fermentation, glycerol fermentation was not affected by SLS. Conversely, the fermentation rate of glycerol in SLS was higher than that of GS. The analysis of microbial diversity revealed that high relative abundances of fermentative microorganisms was identified in SLS. This shows that the reason for the decrease of the glycerol RE in UASB reactor was not related to the degradation mechanism of glycerol by the SLS, but because the SLS triggered the washout of biomass from the UASB reactor.

FTIR spectra showed no significant differences between GS and SLS samples. The biggest difference of peaks between GS and SLS was around at 1635 and 1030 cm^{-1}. The ratio between the absorbance at 1635 cm^{-1} over the absorbance at 1030 cm^{-1} shows the relatively quantity of protein. The ratio was 1.1 for SLS and 0.8 for GS, which indicates that SLS contained relatively more proteins than GS. Proteins are fundamental components of biomolecules. The soluble protein content generally increases after exposure to pollutants. In order to reduce toxic effects, microbes can be induced to synthesize defensive proteins under the stress of hazardous substances (Imlay, 2008; Sabatini et al., 2009). Specifically, Li et al. (2022) showed that excessive extracellular polymeric substances (EPS) production, especially the protein-like substances, was an effective strategy for reducing certain nanoparticles toxicity in anaerobic granular sludge. Since SLS covered GS, SLS first contacted LCFAs and sulfide during glycerol fermentation and sulfate reduction. Thus, compared to GS, SLS had higher exposure to sulfide and LCFAs, which may explain higher protein contents in SLS.

Overall, different methods (SEM, FAMEs, Illumina sequencing and FT-IR) used for SLS analysis showed that SLS was the mixture of biomass and organic/inorganic compounds coming from the impurities contained in crude glycerol and the formation of other intermediate products. With all the information acquired, the formation of SLS was attributed to a combination of factors along the long-term operation, including sulfide production, the cease of methanogenic activity, washout of methanogens, static sludge bed, impurities compounds contained in crude glycerol and the formation of other intermediates.

As a by-product of the biodiesel industry, there are clear advantages in the use of crude glycerol as electron donor such as its large COD content, its availability and low cost among others. However, the presence of impurities may hinder its application unless alternatives to face long-term operation issues are found. Oleszkiewicz and Romanek (1989) reported that the addition of calcium and phosphate promoted sticky, flocculant sludge formation, while granular biomass was formed in the treatment of wastewater supplemented with ferric ions and traces of nickel and cobalt. The use of traces of metallic elements can promote granulation and avoid sludge disintegration and washout of biomass. Increasing the shear force in the reactor could be another strategy for preventing granules flotation (Chen et al., 2014) and crude glycerol by-products accumulation. The increase of shearing forces can be achieved by adding an internal recirculation pump to increase the upflow velocity as well as the use of intermittent pulses of nitrogen from the bottom of the reactor to also avoid bed packing. However, further research is needed in order to assess the viability of such alternatives. Despite the fact that the performance deterioration was mainly attributed to the washout of biomass due to SLS accumulation and consequent sludge flotation, still other factors not considered in the present work such as the change of the metabolic functions of the microbial cultures linked to the SLS accumulation should be investigated.

4. Conclusions

The lack of biogas production in a sulfate-reducing UASB lead to a reduction in the particle size of granular biomass and to the accumulation of a SLS due to the presence of organic compounds and other impurities present in crude glycerol. SLS accumulation also led to sludge flotation, which resulted in an increased washout of sludge. Subsequently, the sulfate RE and glycerol RE in the reactor decreased, and the system performance deteriorated. Sulfate removal capacity and glycerol removal capacity at different heights of the UASB reactor correlated well with the sludge concentration. In the sulfate reduction processes with propionate and glycerol as carbon sources in batch tests, results confirmed that SLS did not change the mechanism of sulfate reduction and glycerol degradation but decreased the rate of sulfate reduction.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Authors acknowledge the Spanish Government, through the project RTI2018-099362-B-C21 MINECO/FEDER, EU, for the financial support provided to perform this research. The first author thanks the China Scholarship Council (CSC, 201706300052) for financial support.

Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2022.11.011.

REFERENCES

Abou-Zeid, D.M., Biebl, H., Spröer, C., Müller, R.J., 2004. *Propionispora hippei* sp. nov., a novel Gram-positive, spore-forming anaerobe that produces propionic acid. Int. J. Syst. Evol. Microbiol. 54, 951–954. doi:10.1099/ijs.0.03054-0.

APHA, 2012. In: Standard Methods for Examination of Water and Wastewater, 5. APHA, AWWA, WEF, pp. 185–186. doi:10.5209/rev_ANHM.2012.v5.n2.40440.

Baba, Y., Tada, C., Watanabe, R., Fukuda, Y., Chida, N., Nakai, Y., 2013. Anaerobic digestion of crude glycerol from biodiesel manufacturing using a large-scale pilot plant: methane production and application of digested sludge as fertilizer. Bioresour. Technol. 140, 342–348. doi:10.1016/j.biortech.2013.04.020.

Bak, F., Pfennig, N., 1991. Sulfate-reducing bacteria in littoral sediment of Lake Constance. FEMS Microbiol. Lett. doi:10.1111/j.1574-6968.1991.tb04696.x.

Bertolino, S.M., Melgaço, L.A., Sá, R.G., Leão, V.A., 2014. Comparing lactate and glycerol as a single-electron donor for sulfate reduction in fluidized bed reactors. Biodegradation 25, 719–733. doi:10.1007/s10532-014-9694-1.

Bertolino, S.M., Rodrigues, I.C.B., Guerra-Sá, R., Aquino, S.F., Leão, V.A., 2012. Implications of volatile fatty acid profile on the metabolic pathway during continuous sulfate reduction. J. Environ. Manage. 103, 15–23. doi:10.1016/J.JENVMAN.2012.02.022.

Biebl, H., Schwab-Hanisch, H., Spröer, C., Lünsdorf, H., 2000. *Propionispora vibrioides*, nov. gen., nov. sp., a new gram-negative, spore-forming anaerobe that ferments sugar alcohols. Arch. Microbiol. 174, 239–247. doi:10.1007/s002030000198.

Chen, H., Ma, C., Yang, G.F., Wang, H.Z., Yu, Z.M., Jin, R.C., 2014. Floatation of flocculent and granular sludge in a high-loaded anammox reactor. Bioresour. Technol. 169, 409–415. doi:10.1016/j.biortech.2014.06.063.

Chen, J., Yan, S., Zhang, X., Tyagi, R.D., Surampalli, R.Y., Valéro, J.R., 2018. Chemical and biological conversion of crude glycerol derived from waste cooking oil to biodiesel. Waste Manag. 71, 164–175. doi:10.1016/j.wasman.2017.10.044.

Cheng, K.K., Zhang, J.A., Liu, D.H., Sun, Y., Liu, H.J., Yang, M.De, et al., 2007. Pilot-scale production of 1,3-propanediol using *Klebsiella pneumoniae*. Process Biochem. 42, 740–744. doi:10.1016/j.procbio.2007.01.001.

Deaver, J.A., Diviesti, K.I., Soni, M.N., Campbell, B.J., Finneran, K.T., Popat, S.C., 2020. Palmitic acid accumulation limits methane production in anaerobic co-digestion of fats, oils and grease with municipal wastewater sludge. Chem. Eng. J. 396, 125235. doi:10.1016/j.cej.2020.125235.

Dinkel, V.G., Frechen, F.B., Dinkel, A.V., Smirnov, Y.Y., Kalyuzhnyi, S.V., 2010. Kinetics of anaerobic biodegradation of glycerol by sulfate-reducing bacteria. Appl. Biochem. Microbiol. 46, 712–718. doi:10.1134/s0003683810070069.

Fehmberger, C., Dos Santos, F.T., Aloisio, C.M., Hermes, E., Zenatti, D.C., Bautitz, I.R., 2020. Effectiveness of incorporation of crude glycerin as a source of labile carbon in the composting of poultry production residues. J. Clean. Prod. 251, 119739. doi:10.1016/j.jclepro.2019.119739.

Fernández-Palacios, E., Lafuente, J., Mora, M., Gabriel, D., 2019. Exploring the performance limits of a sulfidogenic UASB during the long-term use of crude glycerol as electron donor. Sci. Total Environ. 688, 1184–1192. doi:10.1016/j.scitotenv.2019.06.371.

Fernández-Palacios, E., Zhou, X., Mora, M., Gabriel, D., 2021. Microbial diversity dynamics in a methanogenic-sulfidogenic UASB reactor. Int. J. Environ. Res. Public Health 18, 1305. doi:10.3390/ijerph18031305.

Fernández-Palacios, E., 2020. Integrated Assessment of Long-term Sulfidogenesis in UASB Reactors Using Crude Glycerol as Carbon Source PhD thesis. Univ. Autònoma, Barcelona, Spain.

Field, A., 2013. Discovering Statistics Using IBM SPSS Statistics. Sage.

Himmi, E.H., Bories, A., Boussaid, A., Hassani, L., 2000. Propionic acid fermentation of glycerol and glucose by *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii* ssp. *shermanii*. Appl. Microbiol. Biotechnol. 53, 435–440. doi:10.1007/s002530051638.

Hu, S., Luo, X., Wan, C., Li, Y., 2012. Characterization of crude glycerol from biodiesel plants. J. Agric. Food Chem. 60, 5915–5921. doi:10.1021/jf3008629.

Huisman, J.L., Schouten, G., Schultz, C., 2006. Biologically produced sulphide for purification of process streams, effluent treatment and recovery of metals in the metal and mining industry. Hydrometallurgy 83, 106–113. doi:10.1016/j.hydromet.2006.03.017.

Hulshoff Pol, L.W., De Castro Lopes, S.I., Lettinga, G., Lens, P.N.L., 2004. Anaerobic sludge granulation. Water Res. 38, 1376–1389. doi:10.1016/j.watres.2003.12.002.

Hung, C.H., Chang, Y.T., Chang, Y.J., 2011. Roles of microorganisms other than *Clostridium* and *Enterobacter* in anaerobic fermentative biohydrogen production systems - a review. Bioresour. Technol. 102, 8437–8444. doi:10.1016/j.biortech.2011.02.084.

Hwu, C.S., Tseng, S.K., Yuan, C.Y., Kulik, Z., Lettinga, G., 1998. Biosorption of long-chain fatty acids in UASB treatment process. Water Res. 32, 1571–1579. doi:10.1016/S0043-1354(97)00352-7.

Imlay, J.A., 2008. Cellular defenses against superoxide and hydrogen peroxide. Annu. Rev. Biochem. 77, 755–776. doi:10.1146/annurev.biochem.77.061606.161055.

Jeganathan, J., Nakhla, G., Bassi, A., 2006. Long-term performance of high-rate anaerobic reactors for the treatment of oily wastewater. Environ. Sci. Technol. 40, 6466–6472. doi:10.1021/es061071m.

Joubert, W.A., Britz, T.J., 1987. Characterization of aerobic, facultative anaerobic, and anaerobic bacteria in an acidogenic phase reactor and their metabolite formation. Microb. Ecol. 13, 159–168. doi:10.1007/BF02011251.

Jung, H, Kim, J, Lee, C, 2019. Temperature effects on methanogenesis and sulfidogenesis during anaerobic

digestion of sulfur-rich macroalgal biomass in sequencing batch reactors. Microorganisms 7, 682. doi:10.3390/microorganisms7120682.
Kaksonen, A.H., Puhakka, J.A., 2007. Sulfate reduction based bioprocesses for the treatment of acid mine drainage and the recovery of metals. Eng. Life Sci. doi:10.1002/elsc.200720216.
Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41, 1–11. doi:10.1093/nar/gks808.
Kobayashi, T., Xu, K.Q., Chiku, H., 2015. Release of extracellular polymeric substance and disintegration of anaerobic granular sludge under reduced sulfur compounds-rich conditions. Energies 8, 7968–7985. doi:10.3390/en8087968.
Kumar, L.R., Yellapu, S.K., Tyagi, R.D., Zhang, X., 2019. A review on variation in crude glycerol composition, bio-valorization of crude and purified glycerol as carbon source for lipid production. Bioresour. Technol. 293, 122155. doi:10.1016/j.biortech.2019.122155.
Lens, P., Vallero, M., Esposito, G., Zandvoort, M., 2002. Perspectives of sulfate reducing bioreactors in environmental biotechnology. Rev. Environ. Sci. Biotechnol. doi:10.1023/A:1023207921156.
Li, H., Chang, F., Li, Z., Cui, F., 2022. The role of extracellular polymeric substances in the toxicity response of anaerobic granule sludge to different metal oxide nanoparticles. Int. J. Environ. Res. Public Health 19, 5371. doi:10.3390/ijerph19095371.
Liamleam, W., Annachhatre, A.P., 2007. Electron donors for biological sulfate reduction. Biotechnol. Adv. 25, 452–463. doi:10.1016/j.biotechadv.2007.05.002.
Lopes, S.I.C., Wang, X., Capela, M.I., Lens, P.N.L., 2007. Effect of COD/SO_4^{2-} ratio and sulfide on thermophilic (55°C) sulfate reduction during the acidification of sucrose at pH 6. Water Res. 41, 2379–2392. doi:10.1016/j.watres.2007.02.023.
Lu, X., Zhen, G., Chen, M., Kubota, K., Li, Y.Y., 2015a. Biocatalysis conversion of methanol to methane in an upflow anaerobic sludge blanket (UASB) reactor: Long-term performance and inherent deficiencies. Bioresour. Technol. 198, 691–700. doi:10.1016/j.biortech.2015.09.073.
Lu, X., Zhen, G., Estrada, A.L., Chen, M., Ni, J., Hojo, T., Kubota, K., Li, Y.Y., 2015b. Operation performance and granule characterization of upflow anaerobic sludge blanket (UASB) reactor treating wastewater with starch as the sole carbon source. Bioresour. Technol. 180, 264–273. doi:10.1016/j.biortech.2015.01.010.
Mahmoud, N., Zeeman, G., Gijzen, H., Lettinga, G., 2004. Anaerobic sewage treatment in a one-stage UASB reactor and a combined UASB-Digester system. Water Res. 38, 2348–2358. doi:10.1016/j.watres.2004.01.041.
Mora, M., Fernández-Palacios, E., Guimerà, X., Lafuente, J., Gamisans, X., Gabriel, D., 2020a. Feasibility of S-rich streams valorization through a two-step biosulfur production process. Chemosphere 253, 126734. doi:10.1016/j.chemosphere.2020.126734.
Mora, M., Lafuente, J., Gabriel, D., 2020b. Influence of crude glycerol load and pH shocks on the granulation and microbial diversity of a sulfidogenic upflow anaerobic sludge blanket reactor. Process Saf. Environ. Prot. 133, 159–168. doi:10.1016/j.psep.2019.11.005.
Mora, M., Lafuente, J., Gabriel, D., 2018. Screening of biological sulfate reduction conditions for sulfidogenesis promotion using a methanogenic granular sludge. Chemosphere 210, 557–566. doi:10.1016/j.chemosphere.2018.07.025.
Moscoviz, R., Trably, E., Bernet, N., 2018. Electro-fermentation triggering population selection in mixed-culture glycerol fermentation. Microb. Biotechnol. 11, 74–83. doi:10.1111/1751-7915.12747.
Muyzer, G., Stams, A.J.M., 2008. The ecology and biotechnology of sulphate-reducing bacteria. Nat. Rev. Microbiol. 6, 441–454. doi:10.1038/nrmicro1892.
O'Flaherty, V., Mahony, T., O'Kennedy, R., Colleran, E., 1998. Effect of pH on growth kinetics and sulphide toxicity thresholds of a range of methanogenic, syntrophic and sulphate-reducing bacteria. Process Biochem. 33, 555–569. doi:10.1016/S0032-9592(98)00018-1.
Odom, J.M., Peck, H.D., 1981. Hydrogen cycling as a general mechanism for energy coupling in the sulfate-reducing bacteria, *Desulfovibrio* sp. FEMS Microbiol. Lett. 12, 47–50. doi:10.1111/j.1574-6968.1981.tb07609.x.
Oleszkiewicz, J.A., Romanek, A., 1989. Granulation in anaerobic sludge bed reactors treating food industry wastes. Biol. Wastes 27, 217–235. doi:10.1016/0269-7483(89)90003-7.
Omil, F., Lens, P., Hulshoff Pol, L.W., Lettinga, G., 1997a. Characterization of biomass from a sulfidogenic, volatile fatty acid-degrading granular sludge reactor. Enzyme Microb. Technol. 20, 229–236. doi:10.1016/S0141-0229(96)00119-6.
Omil, F., Oude Elferink, S.J.W.H., Lens, P., Hulshoff Pol, L.W., Lettinga, G., 1997b. Effect of the inoculation with *Desulforhabdus amnigenus* and pH or O_2 shocks on the competition between sulphate reducing and methanogenic bacteria in an acetate fed UASB reactor. Bioresour. Technol. 60, 113–122. doi:10.1016/S0960-8524(97)00014-X.
Ozgun, H., Ersahin, M.E., Zhou, Z., Tao, Y., Spanjers, H., van Lier, J.B., 2019. Comparative evaluation of the sludge characteristics along the height of upflow anaerobic sludge blanket coupled ultrafiltration systems. Biomass Bioenergy 125, 114–122. doi:10.1016/j.biombioe.2019.04.001.
Pereira, M.A., Pires, O.C., Mota, M., Alves, M.M., 2005. Anaerobic biodegradation of oleic and palmitic acids: evidence of mass transfer limitations caused by long chain fatty acid accumulation onto the anaerobic sludge. Biotechnol. Bioeng. 92, 15–23. doi:10.1002/bit.20548.
Qatibi, A.I., Nivière, V., Garcia, J.L., 1991. *Desulfovibrio alcoholovorans* sp. nov., a sulfate-reducing bacterium able to grow on glycerol, 1,2- and 1,3-propanediol. Arch. Microbiol. 155, 143–148. doi:10.1007/BF00248608.
Rizvi, H., Ahmad, N., Abbas, F., Bukhari, I.H., Yasar, A., Ali, S., et al., 2015. Start-up of UASB reactors treating municipal wastewater and effect of temperature/sludge age and hydraulic retention time (HRT) on its performance. Arab. J. Chem. 8, 780–786. doi:10.1016/j.arabjc.2013.12.016.
Sabatini, S.E., Juárez, Á.B., Eppis, M.R., Bianchi, L., Luquet, C.M., Ríos de Molina, M., del, C., 2009. Oxidative stress and antioxidant defenses in two green microalgae exposed to copper. Ecotoxicol. Environ. Saf. 72, 1200–1206. doi:10.1016/j.ecoenv.2009.01.003.
Sarti, A., Zaiat, M., 2011. Anaerobic treatment of sulfate-rich wastewater in an anaerobic sequential batch reactor (AnSBR) using butanol as the carbon source. J. Environ. Manage. 92, 1537–1541. doi:10.1016/j.jenvman.2011.01.009.
Shin, H.S., Oh, S.E., Bae, B.U., 1996. Competition between SRB and MPB according to temperature change in the anaerobic treatment of tannery wastes containing high sulfate. Environ. Technol. 17, 361–370. doi:10.1080/09593331708616395.
Silva, S.A., Salvador, A.F., Cavaleiro, A.J., Pereira, M.A., Stams, A.J.M., Alves, M.M., et al., 2016. Toxicity of long chain fatty acids towards acetate conversion by *Methanosaeta concilii* and *Methanosarcina mazei*. Microb. Biotechnol. 9, 514–518. doi:10.1111/1751-7915.12365.
Vallero, M.V.G., Camarero, E., Lettinga, G., Lens, P.N.L., 2004. Thermophilic (55-65°C) and extreme thermophilic (70-80°C) sulfate reduction in methanol and formate-fed UASB reactors. Biotechnol. Prog. 20, 1382–1392. doi:10.1021/bp034329a.
Vallero, M.V.G., Hulshoff Pol, L.W., Lettinga, G., Lens, P.N.L., 2003. Effect of NaCl on thermophilic (55°C) methanol degradation in

sulfate reducing granular sludge reactors. Water Res. 37, 2269–2280. doi:10.1016/S0043-1354(03)00024-1.
Viana, M.B., Freitas, A.V., Leitão, R.C., Pinto, G.A.S., Santaella, S.T., 2012. Anaerobic digestion of crude glycerol: a review. Environ. Technol. Rev. 1, 81–92. doi:10.1080/09593330.2012.692723.
Weijma, J., Stams, A.J.M., Hulshoff Pol, L.W., Lettinga, G., 2000. Thermophilic sulfate reduction and methanogenesis with methanol in a high rate anaerobic reactor. Biotechnol. Bioeng. 67, 354–363. doi:10.1002/(SICI)1097-0290(20000205)67:3⟨354::AID-BIT12⟩3.0.CO;2-X.
Westerholm, M., Isaksson, S., Karlsson Lindsjö, O., Schnürer, A., 2018. Microbial community adaptability to altered temperature conditions determines the potential for process optimisation in biogas production. Appl. Energy 226, 838–848. doi:10.1016/j.apenergy.2018.06.045.
Wu, J., Afridi, Z.U.R., Cao, Z.P., Zhang, Z.L., Poncin, S., Li, H.Z., et al., 2016. Size effect of anaerobic granular sludge on biogas production: a micro scale study. Bioresour. Technol. 202, 165–171. doi:10.1016/j.biortech.2015.12.006.
Wu, J., Niu, Q., Li, L., Hu, Y., Mribet, C., Hojo, T., et al., 2018. A gradual change between methanogenesis and sulfidogenesis during a long-term UASB treatment of sulfate-rich chemical wastewater. Sci. Total Environ. 636, 168–176. doi:10.1016/j.scitotenv.2018.04.172.
Zeng, D., Yin, Q., Du, Q., Wu, G., 2019. System performance and microbial community in ethanol-fed anaerobic reactors acclimated with different organic carbon to sulfate ratios. Bioresour. Technol. 278, 34–42. doi:10.1016/j.biortech.2019.01.047.
Zhou, X., Fernández-Palacios, E., Dorado, A.D., Gamisans, X., Gabriel, D., 2022. Assessing main process mechanism and rates of sulfate reduction by granular biomass fed with glycerol under sulfidogenic conditions. Chemosphere 286, 131649. doi:10.1016/J.CHEMOSPHERE.2021.131649.