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Assessing main process mechanism and rates of sulfate reduction by granular biomass fed with glycerol under sulfidogenic conditions

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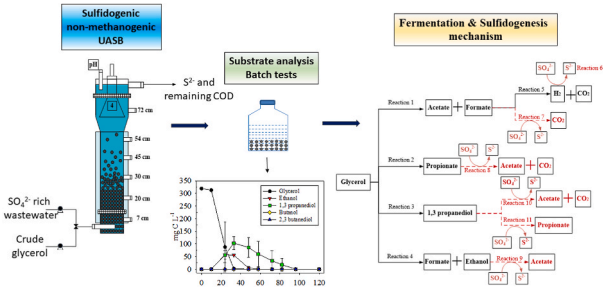
HIGHLIGHTS

- The mechanisms of sulfate reduction using glycerol were established.
- Ethanol and 1,3-propanediol were the main electron donors in sulfate reduction.
- Incomplete-oxidizing SRB dominated under sulfidogenic conditions.
- SRB preferentially used simple intermediate products except for acetate.

GRAPHICAL ABSTRACT



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ABSTRACT

Sulfate-reducing bioreactors for sulfide production are the initial stage of processes targeting elemental sulfur recovery from sulfate-rich effluents. In this work, the principal reactions involved in glycerol fermentation and sulfate reduction using glycerol and its fermentation products as electron donors were assessed together with their specific consumption/production rates. A battery of batch activity tests with and without sulfate were performed with glycerol and with each fermentation product using a non-methanogenic but sulfidogenic granular sludge from an up-flow anaerobic sludge blanket (UASB) reactor operated under long-term while fed with crude glycerol. As a result, a mechanistic approach based on the experimental observations is proposed in this work. Glycerol was mainly fermented to 1,3-propanediol, ethanol, formate, propionate and acetate by fermentative bacteria. All organic intermediates were found to be further used by sulfate reducing bacteria (SRB) for sulfate reduction except for acetate. The most abundant genus detected under sulfidogenic conditions were *Propionispora* (15.2%), *Dysgonomonas* (13.2%), *Desulfobulbus* (11.6%) and *Desulfovibrio* (10.8%). The last two SRB genera accounted for 22.4% of the total amount of retrieved sequences, which were probably performing an incomplete oxidation of the carbon source in the sulfidogenic UASB reactor. As single substrates, specific sulfate reduction rates (SRRs) using low molecular weight (MW) carbon sources (formate and ethanol) were 39% higher than those using high-MW ones (propionate, 1,3-propanediol and butanol). However, SRRs in glycerol-fed tests showed that 1,3-propanediol played a major role in sulfate reduction in addition to formate and ethanol.

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1. Introduction

Sulfate is an anion widely present in natural environments. However, high concentrations of sulfate can be toxic to aquatic life (Karjalainen et al., 2021). In aquatic environments, sulfate can be also reduced to hydrogen sulfide by sulfate-reducing bacteria (SRB) under anaerobic conditions generating further problems since hydrogen sulfide is poisonous and corrosive. Many industrial sites including pulp and paper and mining industries, tanneries, fermenting plants and thermal power plants generate flue gases or wastewaters containing large amounts of sulfur, mainly as sulfate, that require further treatment. As an alternative to costly physical-chemical technologies, environmentally friendly, biological processes arose recently, some considering the valorization of S-rich emissions into elemental sulfur (biosulfur) (Mora et al., 2020a), a value-added product currently obtained from the petrochemical industry. Since microbial communities able to reduce sulfate directly to biosulfur have not been described yet, one biological-based alternative relies on a two-stage process (Mora et al., 2020a). First, sulfate is reduced to sulfide in an upflow anaerobic sludge blanket (UASB) bioreactor, and subsequently, sulfide is partially oxidized to elemental sulfur in a second bioreactor under microaerobic or anoxic conditions. The technical feasibility of biosulfur recovery in a two-stage bioscrubber has been demonstrated previously in the SONOVA process (Mora et al., 2020a) despite some operational problems in the long-term leading to biomass losses in the anaerobic stage (Fernández-Palacios et al., 2021). However, further fundamental analysis is warranted to reveal the underlying mechanisms in the UASB to develop a more robust, transferable technology to field applications.

UASB reactors are widely used in anaerobic digestion and have also been reported as an appropriate technology for sulfate reduction using different carbon sources, including methanol (Weijma and Stams, 2001), ethanol (Wu et al., 2018), sucrose (Weijma and Stams, 2001) and crude glycerol (Fernández-Palacios et al., 2019). As an alternative to other expensive electron donors, crude glycerol is a by-product mainly produced in the biodiesel production industry with a high market potential. It was estimated that world glycerol production would reach 2.66 million tons in 2020 (Kumar et al., 2019). The composition of crude glycerol varies depending on the process, containing around 70–98% of glycerol plus some impurities including water, long-chain fatty acids, fatty acid methyl esters, salts, methanol, soap, and ashes (Angeloni et al., 2016; Viana et al., 2012; Vivek et al., 2017).

Metabolic pathways of glycerol fermentation in anaerobic digestion have been clearly described by a combination of reductive and oxidative reactions (Viana et al., 2012). From one side, glycerol is first dehydrated to 3-hydroxypropionaldehyde that is further reduced to 1,3-propanediol by the enzyme 1,3-propanediol dehydrogenase. On the other hand, via an oxidative pathway, glycerol is also converted to phosphoenolpyruvate, which subsequently produces propionate and pyruvate. Pyruvate can further produce other compounds such as n-butanol, 2,3-butanediol, ethanol, lactate, butyrate, formate, acetate, hydrogen and CO_2 depending on the microbial cultures and environmental conditions. In addition to H_2, most of the abovementioned organic compounds can be used as electron donors by SRB to reduce sulfate to sulfide.

The reduction of sulfate to sulfide by SRB follows both assimilative and dissimilative metabolisms. Dissimilatory sulfate reduction is the main pathway to produce hydrogen sulfide (Madigan et al., 1997) by SRB using sulfate as the terminal electron acceptor either through autotrophic or heterotrophic metabolisms (Lens and Kuenen, 2001). Autotrophic SRB use CO_2 as carbon source and H_2 as electron donor to reduce sulfate while heterotrophic SRB can use a wide variety of organic compounds as electron donor including organic acids (formic, acetic, propionic, butyric, pyruvic, lactic, etc.) and alcohols (methanol, ethanol, butanol etc.). Heterotrophic SRB include incomplete oxidizers (that partly oxidize organic compounds to acetate), and complete oxidizers (that entirely oxidize organic compounds to CO_2).

Previous studies have reported that glycerol as electron donor can be directly oxidized to acetate and bicarbonate in the sulfate reduction process (Bertolino et al., 2014; Dinkel et al., 2010; Qatibi et al., 1991a). However, the TOC imbalance found in these works was suggested to be caused by other intermediate products. Several studies have shown that other products are produced when glycerol is used as electron donor to reduce sulfate. As examples, 3-hydroxypropionate (Qatibi et al., 1998) and butyrate (Bertolino et al., 2014) were produced in glycerol fermentation coupled to sulfate reduction. It was also found that glycerol was incompletely oxidized to acetate, lactate, and 1,3-propanediol under low pH conditions (Santos et al., 2018). However, the mechanism of the sulfate reduction process, which is also culture dependent, is still unclear as most of literature have only shown the oxidized products of glycerol during this process.

The purpose of this work was to assess the main mechanism and the process rates of glycerol fermentation and sulfate reduction of a sulfidogenic granular sludge enriched in an UASB fed with crude glycerol under long-term operating conditions without methane production. To this aim, microbial activity was assessed in batch tests with a range of electron donors including glycerol, alcohols and volatile fatty acids (VFAs) with and without the presence of sulfate.

2. Materials and methods

2.1. Granular biomass growth conditions

A laboratory-scale UASB reactor as described in previous works (Fernández-Palacios et al., 2019; Mora et al., 2020a) was set up and inoculated with granular sludge from a full-scale UASB from a paper recycling industry (Spain), which was mainly used for methane production. The lab-scale UASB was operated to promote sulfidogenic, non-methanogenic conditions as shown in supplementary material (Fig. S1). In order to minimize the effect of changing conditions, the sulfate inlet concentration was set to 250 mg $S–SO_4^{2-}$ L^{-1} while the crude glycerol to sulfate ratio (TOC/$S–SO_4^{2-}$) was steadily maintained at 1.49 ± 0.31 g C/g $S–SO_4^{2-}$. These conditions were selected according to the period of maximum sulfate reducing and organic removal efficiencies, according to Fernández-Palacios et al. (2019). The mineral medium (MM) composition fed to the UASB was (in g L^{-1}): NH_4Cl (0.2), K_2HPO_4 (3), and Na_2SO_4 (1.15) dissolved in tap water. The pH of the MM was adjusted to 8.5–8.8 using 2 M NaOH. The MM flow was mixed with the carbon source (crude glycerol) flowrate and pumped into the UASB from the bottom of the reactor. The temperature was controlled at 35 °C with a water bath and the up-flow velocity was set at 0.25 m h^{-1}. The UASB reactor was operated stably for 639 days under the conditions described above. Also, the redox potential (ORP) in the reactor was monitored throughout the operation with an ORP probe (pH 5350, Crison, Spain) on top of the reactor close to the liquid outlet. The ORP varied from −460 mV to −540 mV along the operation, indicating that dissolved oxygen did not penetrate into the UASB.

2.2. Batch activity tests

In order to identify the main mechanisms of the granular sludge using glycerol as electron donor to reduce sulfate in the sulfidogenic UASB, granular sludge was taken from the UASB after 431, 549 and 578 days of operation, when no methane was produced. The sludge withdrawn from the UASB on different days was tested with different carbon sources. Pure glycerol, ethanol, 1,3-propanediol, butanol and 2,3-butanediol were tested with the sludge collected on day 431. Formate was tested with the sludge collected on day 549, while acetate and propionate were tested with the sludge collected on day 578. Sludge samples were first rinsed with MM (without sulfate) and then transferred into 250 ml serum bottles, which were afterwards fed with 150 ml of MM. In all cases, a control group without sulfate and an experimental group were evaluated. The control group consisted of MM (without sulfate) supplied with different carbon sources only, while in the experimental

group sulfate was added to the MM and the carbon source. The initial carbon concentration ranged from 318 to 372 mg C L^{-1} in both the control and the experimental groups. The initial sulfate concentration of the experimental group was set to 240 ± 4 mg S–SO_4^{2-} L^{-1}, and the TOC/S–SO_4^{2-} ratio was 1.4 ± 0.1 g C/g S–SO_4^{2-}. After adding MM and the granular sludge, the pH was adjusted to 8–8.5 by adding either 2 M NaOH or 2 M HCl. The pH was not controlled during activity tests, but it was measured at the beginning and at the end of each test with a pH probe (pH 5333, Crison, Spain). The gas phase was exchanged with N_2 and bottles were instantly capped with rubber stoppers and aluminum caps. Bottles were cultivated in a shaker (NB-T205, *N*-Biotek, Korea) at a constant temperature of 35 ± 1 °C and a stirring rate of 150 rpm. The incubation time was between 96 and 120 h depending on the test. Volatile suspended solids (VSS) in batch tests were obtained at the end of each experiment and ranged from 125 to 390 mg VSS L^{-1}. All batch tests were carried out with two replicates.

2.3. Analytical methods

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). Total dissolved sulfide (TDS) was analyzed by a sulfide selective electrode (9616BNWP, Thermo Scientific, MA, USA) connected to a benchtop meter (Symphony, VWR, Germany). Before TDS measurement, samples were diluted 20 times with a sulfide antioxidant buffer (SAOB) containing (g L^{-1}): ascorbic acid (35), EDTA (67) and NaOH (80).

Volatile fatty acids (VFAs) and alcohols 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 mM H_2SO_4 mobile phase at a flow rate of 0.5 mL min^{-1}. Total organic carbon (TOC), total inorganic carbon (TIC) and total carbon (TC) were determined in a TOC analyzer (multi N/C 2100S, Analytikjena, Germany). All samples were filtered by 0.22 μm (Millipore, USA) before analysis.

CH_4, CO_2 and H_2 from test bottles headspace were analyzed by gas chromatography (7820A, Agilent Technologies, USA) equipped with a detector (TCD) and a capillary column (Al_2O_3 PLOT: 50 m × 0.53 mm). H_2S was analyzed by gas chromatography (HP 5890A GC, Hewlett Packard, USA) equipped with a thermal conductivity detector and a Porapak Q column. Before measuring the gas composition in the headspace of each bottle, pressure was measured by a manometer (SMC ISE30A-01-P, Japan).

Volumetric consumption/production rates of the measured species were calculated by the concentration changes between two consecutive sampling events and, then, averaged for the whole length of the specific period evaluated. VSS were measured according to Standard Methods (APHA, 2012) to calculate specific rates.

2.4. Illumina sequencing analysis

Identification of the microbial population was performed using Illumina platform of samples collected on day 538 of the long-term operation of the UASB. Genomic DNA was extracted by applying the protocol of PowerSoil™ DNA isolation kit (MoBio Laboratories, USA) following the supplier's instructions. The quantity and quality of the extracted DNA were assessed by using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Then, DNA samples were preserved at −20 °C for further analysis. Sequencing analyses were performed by "Genomic and Bioinformatics service" at the Universitat Autònoma de Barcelona. Amplicon sequencing that targets the V3–V4 hypervariable regions (HVRs) of the 16S rRNA gene on Illumina MiSeq platform were carried out using the universal primers 341F (5′- CCT ACG GGN GGC WGC AG-3′) and 805R (5′- GAC TAC HVG GGT ATC TAA TCC -3′). The database used for the classification of organisms is based on the Greengenes database (http://greengenes.lbl.gov/).

3. Results and discussion

3.1. Activity tests with glycerol as sole external carbon source

Long-term operation of the UASB reactor was performed under constant sulfate and organic loading rates for 639 days. The experimental results and discussion of the UASB performance and the evolution of the microbial diversity can be found elsewhere (Fernández-Palacios et al. (2021). In short, after operating the UASB reactor for 200 days, methane production decreased significantly concomitantly with VFAs production. At a TOC/S–SO_4^{2-} ratio of 1.49 ± 0.31, the reactor had undergone a progressive washout of methanogens, including *Methanosaeta, Methanobacteria*, and *Methanomicrobiales*. SRB completely outcompeted methanogens after 230 days of operation, in which *Desulfobulbus* and *Desulfovibrio* were found the main sulfate-reducing genus. Through the treatment of sulfate-rich wastewater, the methanogenic sludge was shifted into a sulfidogenic sludge. Activity tests presented herein were performed to study the sulfate reduction mechanism using glycerol as carbon source using sludge collected from the reactor when it was performing under sulfidogenic conditions. From day 200 to day 400, only VFAs were considered as the intermediate products of glycerol degradation, but a significant carbon imbalance was found during the process of glycerol fermentation. Therefore, other intermediate metabolites were considered in batch tests after day 400, such as ethanol and 1,3-propanediol.

3.1.1. Glycerol fermentation process

Profiles for carbon and sulfur species using pure glycerol as the only carbon source externally added to the serum bottles with and without the presence of sulfate are shown in Fig. 1. In the control group (Fig. 1A and B), only glycerol was added with an initial granular sludge concentration of 230 mg VSS L^{-1}. The 120-h culture in the control test was divided into three phases: a lag phase (0–10 h), a quick glycerol uptake phase (10–48 h), and a glycerol-free phase mediated by several intermediates (48–120 h). After the lag phase (10 h), glycerol began to be consumed while ethanol, 1,3-propanediol, formate, acetate and propionate accumulated. Along this second period, glycerol was consumed at a rate of 49.9 ± 13.7 mg C g VSS^{-1} h^{-1} and ethanol, 1,3-propanediol, formate, acetate and propionate were accumulated at rates of 2.1 ± 2.9, 30.3 ± 8.2, 1.6 ± 2.4, 6.2 ± 3.0 and 0.5 ± 0.7 mg C g VSS^{-1} h^{-1}, respectively. At the end of the second phase (48 h), glycerol had already been completely converted to ethanol, 1,3-propanediol, formate, acetate, propionate and inorganic carbon, accounting for 4.5 ± 0%, 57.5 ± 0.6%, 3.9 ± 1.1%, 12.2 ± 2.7%, 1.1 ± 1.5%, and 4.0 ± 0.9% of the initial glycerol content, respectively. During the last phase (48–120 h), 1,3-propanediol and inorganic carbon were maintained at 195 and 23 mg C L^{-1}, respectively. All formate (53.6 mg C L^{-1}) was completely consumed while butyrate increased from 0 to 7.3 mg C L^{-1} and propionate increased from 3.5 to 17.6 mg C L^{-1}. The pH decreased from 8.4 to 7.2 concomitantly with VFAs production.

Results obtained through Illumina sequencing analysis of the 16S rRNA with the sludge sample collected on day 538 from the UASB are presented in supplementary material (Fig. S2). The most abundant genus detected were *Propionispora* (15.2%), *Dysgonomonas* (13.2%), *Desulfobulbus* (11.6%) and *Desulfovibrio* (10.8%). Bacteria from the genus *Dysgonomonas* are obligately anaerobic also known for their fermentative activity while *Propionispora* is a genus of anaerobic fermentative bacteria that typically use glycerol, and other carbohydrates, fermenting them to produce propionic, acetic acid, CO_2 and H_2 (Abou-Zeid et al., 2004). Major fermentation products are usually butyric and acetic acid while propionic, isovaleric, isobutyric and phenylacetic acids may also be produced (Sakamoto, 2014). Additionally, *Klebsiella* (2.1%) was also detected. The metabolism of *Klebsiella pneumoniae* with glycerol has also been described as follows: a) glycerol is converted to ethanol and formate (Jarvis et al., 1997); b) glycerol is degraded into acetate and formate (Zeng et al., 1993); c) lactate, acetate, ethanol and 1,

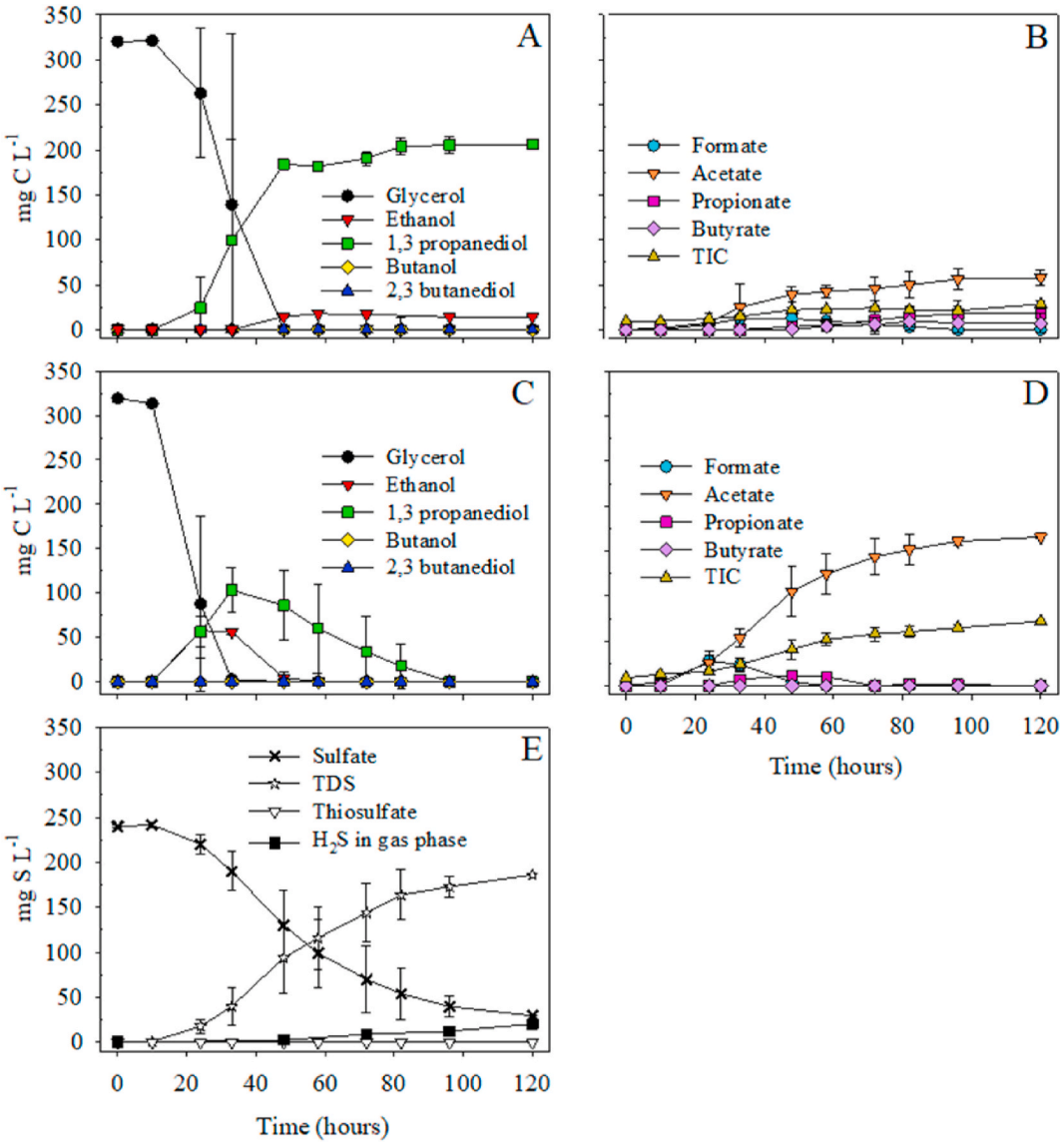

Fig. 1. Anaerobic bioconversion of pure glycerol in serum bottles without sulfate (A, B) and with sulfate (C, D, E) showing the glycerol consumption and the concentration profiles for alcohols (A, C), volatile fatty acids and inorganic carbon (B, D) and sulfur species (E). Error bars represent standard deviations of duplicate tests. TIC represents total inorganic carbon.

3-propanediol are produced as end products (Cheng et al., 2007). According to the products of glycerol shown in Fig. 1A and B and the metabolic pathways of glycerol mentioned above, the stoichiometry of the reactions involved in glycerol fermentation are shown in Table 1.

3.1.2. *Sulfate reduction in glycerol-fed batch tests*

The profiles obtained for the experimental group where glycerol and sulfate were added are shown in Fig. 1C for alcohols, in Fig. 1D for VFAs and in Fig. 1E for sulfur species. The granular sludge concentration in the experimental bottles was 390 mg VSS L^{-1}. The 120-h culture in the experimental test were also divided into three phases: the lag phase (0–10 h), the glycerol fermentation phase (10–33 h) and a secondary carbon sources consumption/production phase (33–120 h). Similar to the control group, the granular sludge in the experimental group also experienced a 10 h lag phase with neither glycerol fermentation nor sulfate reduction. After the lag phase, the specific rates along the test with sulfate were assessed. In the second phase from 10 to 33 h, glycerol was consumed at a rate of 33.1 ± 12.1 mg C g VSS^{-1} h^{-1}, while ethanol, 1,3-propanediol, formate, acetate and propionate accumulated at rates of 5.0 ± 7.9, 11.9 ± 2.1, 2.0 ± 4.5, 5.9 ± 2.7 and 1.0 ± 1.4 mg C g VSS^{-1} h^{-1}, respectively. Sulfate was reduced at a rate of 6.3 ± 3.4 mg S g VSS^{-1} h^{-1} along this phase. At the end of the second phase (33 h), glycerol was completely consumed while ethanol, 1,3-propanediol, formate, acetate, propionate and inorganic carbon accumulated, accounting for 17.5 ± 0.8%, 32.3 ± 8.0%, 7.4 ± 2.2%, 16.7 ± 3.2%, 2.3 ± 0.0%, and 4.5 ± 2.5% of the initial glycerol content, respectively. In the third phase, when there was no glycerol, a net alcohols and VFAs consumption was observed, coupled to sulfate reduction and a significant accumulation of acetate and inorganic carbon. From 33 to 96 h, the sulfate reduction rate (SRR) gradually decreased from 10.4 mg S g VSS^{-1} h^{-1} (33–48 h) to 2.6 mg S g VSS^{-1} h^{-1} (82–96 h). Ethanol, 1,3-propanediol, formate and propionate were completely consumed at a rate of 8.9 ± 0.6, 4.4 ± 1.5, 1.9 ± 1.2 and 1.2 ± 1.0 mg C g VSS^{-1} h^{-1}, respectively. Almost no activity was observed after 96 h of test.

Bertolino et al. (2014) found that acetate and carbonate were the terminal products of glycerol oxidation under sulfidogenic conditions at a sulfate loading rate of 4.7 kg m^{-3} d^{-1} but pointed out that some intermediate products in the degradation process of glycerol were not measured but probably consumed during sulfate reduction. In addition, the production of formate, ethanol and 1,3-propanediol have also been

Table 1
Fermentation and sulfidogenic reactions involved in anaerobic digestion.

Stoichiometric equation	Eq.	Reference
Fermentation reaction		
$C_3H_8O_3(\text{glycerol}) + H_2O + 2NAD^+ \rightarrow C_2H_4O_2(\text{acetic acid}) + HCOOH + 2NADH + 2H^+$	(1)	Zeng et al. (1993)
$C_3H_8O_3(\text{glycerol}) \rightarrow C_3H_6O_2(\text{propionic acid}) + H_2O$	(2)	Schauder and Schink (1989)
$C_3H_8O_3(\text{glycerol}) + NADH + H^+ \rightarrow C_3H_8O_2(1,3-\text{propanediol}) + NAD^+ + H_2O$	(3)	Sittijunda and Reungsang (2017)
$C_3H_8O_3(\text{glycerol}) + NAD^+ \rightarrow C_2H_6O(\text{ethanol}) + HCOOH + NADH + H^+$	(4)	Sittijunda and Reungsang (2017)
$HCOOH \rightarrow CO_2 + H_2$	(5)	Bijmans et al. (2008)
Sulfidogenic reaction		
$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	(6)	Muyzer and Stams (2008)
$4HCOO^- + SO_4^{2-} + H^+ \rightarrow HS^- + 4HCO_3^-$	(7)	Liamleam and Annachhatre (2007)
$C_3H_5O_2^-(\text{propionate}) + 0.75SO_4^{2-} \rightarrow C_2H_3O_2^-(\text{acetate}) + HCO_3^- + 0.75HS^- + 0.25H^+$	(8)	Luis (2018)
$C_2H_6O(\text{ethanol}) + 0.5SO_4^{2-} \rightarrow 0.5HS^- + C_2H_3O_2^-(\text{acetate}) + H_2O + 0.5H^+$	(9)	Wu et al. (2018)
$C_3H_8O_2(1,3-\text{propanediol}) + SO_4^{2-} \rightarrow C_2H_3O_2^-(\text{acetate}) + HS^- + HCO_3^- + H_2O + H^+$	(10)	Qatibi et al. (1991b)

previously reported in the fermentation of glycerol (Jarvis et al., 1997; Katarzyna et al., 2011; Sittijunda and Reungsang, 2017). At the same time, these compounds could also act as intermediate products under sulfidogenic conditions. Ethanol, 1,3-propanediol, formate and propionate have been reported as suitable electron donors in biological sulfate reduction processes applied in different bioreactors, such as UASB and expanded granular sludge bed reactors (De Smul and Verstraete, 1999; Qatibi et al., 1991b; Vallero et al., 2004a, 2004b; Vallero et al., 2004a, 2004b; Wu et al., 2018). The stoichiometry of the reactions involved in sulfate reduction by non-methanogenic but sulfidogenic biomass is shown in Table 1. Results in Fig. 1 confirmed that intermediate reduced organic carbon sources were used as electron donors in sulfate reduction processes. However, data was not sufficient to clearly identify which specific electron donors were used and their SRRs. As an example, when there was no glycerol fermentation, formate was consumed with and without the presence of sulfate (Fig. 1B and D). In the absence of sulfate, formate was consumed because of anaerobic fermentation. However, in the presence of sulfate, the consumption of formate cannot be interpreted as the direct attribution to anaerobic fermentation or its use as an electron donor by SRB. Consequently, separate experiments with individual organic compounds were required to confirm the metabolic pathway of sulfate reduction using ethanol, 1,3-propanediol, formate and propionate (see section 3.2).

The TOC removal (0 h–120 h) in the absence and presence of sulfate were 5.7% and 19.0%, respectively, indicating that SRB played a major role in removing organics. The percentage of relative abundance of SRB on day 538 in the UASB could be considered as the sum of genus *Desulfobulbus* and *Desulfovibrio* accounting for a 22.4% of the total amount of sequences detected. Most bacteria that belong to the genus *Desulfobulbus* can oxidize propionate to acetate in the presence of sulfate and ferment pyruvate and lactate to a mixture of acetate and propionate (El Houari et al., 2017). This genus can also use lactate, pyruvate, ethanol or propanol as carbon sources and also as electron donors for anaerobic respiration, oxidizing them incompletely to acetate. According to Rabus et al. (2013), *Desulfobulbus* and *Desulfovibrio* are not able to completely oxidize organic electron donors, therefore they perform an incomplete oxidation to acetate as end-product. *Desulfobulbus and Desulfovibrio* do not utilize acetate as electron donor even if some species of both genera are able to use H_2 as electron donor to reduce sulfate to sulfide (Rabus et al., 2013). *Desulfovibrio* spp. only carry out an incomplete oxidation of substrates and they are able to excrete acetate as end-product (Rabus et al., 2013). This could indeed explain the accumulation of acetate in the experimental group indicating that SRB found in the UASB reactor performed an incomplete oxidization of the carbon sources used.

By comparing the consumption rate of glycerol during the glycerol fermentation phase in the control group (10–48 h) and the experimental group (10–33 h), it was found that the consumption rate of glycerol in the control group was 29.4 ± 8.1 mg C L^{-1} h^{-1}, similar to that in the experimental group (32.7 ± 12.0 mg C L^{-1} h^{-1}). This indicates that glycerol was not probably used by SRB for sulfate reduction but that glycerol was first fermented by granular sludge to produce secondary carbon substrates, and then SRB used these carbon sources to reduce sulfate. Therefore, it was necessary to explore the sulfate reduction mediated by other electron donors formed during glycerol fermentation in order to understand the mechanism of sulfate reduction by the granular sludge.

3.1.3. Mass balances

The carbon mass balance of the glycerol-added serum bottle experiments is presented in the supplementary material section (without sulfate, Fig. S3A, and with sulfate, Fig. S3B). The sum of carbon was calculated as the sum of volatile fatty acids and alcohols. In the absence of sulfate, the carbon imbalance was 10.1 ± 3.0% between TOC and the sum of carbon species measured (Fig. S3A) from 10 to 120 h. This could be explained by the presence of some other organic carbon species not monitored as well as to biomass growth. Butyrate and propionate accumulation started only after glycerol depletion, which may be due to the accumulation of phosphoenolpyruvate during the fermentation of glycerol (Viana et al., 2012). Phosphoenolpyruvate was subsequently oxidized after 48 h. Moreover, via a reductive pathway, glycerol not only produces 1,3-propanediol but 3-hydroxypropionate, a metabolite of glycerol fermentation (Qatibi et al., 1998). Therefore, these possible intermediate metabolites of glycerol that were not measured may contribute to the 10.1% carbon imbalance. In the presence of sulfate, the carbon imbalance between TOC and the sum of all the rest of carbon species was 18.5 ± 6.3% (Fig. S3B). Compared with the carbon imbalance without sulfate (10.1%), the increase in the imbalance was probably due to the formation of volatile organic sulfur compounds (VOSC). It has been reported that VOSC are produced in the anaerobic digestion of organic wastes (Papurello et al., 2012) as well as propylene glycol and glycerol (Trabue et al., 2007). Compounds such as methanethiol and dimethyl sulfide are formed by the degradation of sulfur-containing amino acids or sulfide methylation in anaerobic digestion (Lomans et al., 2002). As shown in Fig. 1E, the concentration of H_2S in the gas phase measured at 120 h was 20.1 mg S L^{-1}. Compared with the TDS concentration measured in the liquid, the H_2S concentration in the gas phase accounted only for 6.6% of the total S fed. The sulfur mass balance was calculated by monitoring the sulfate, thiosulfate and TDS content in the liquid and the hydrogen sulfide content in the gas phase at the beginning and at the end of each test. On average, a 4.7% sulfur imbalance was observed between the S mass at 0 h and 120 h, which may be caused by the formation of organic sulfur compounds.

The sulfur and carbon mass balances of the UASB reactor are shown in Fig. S1C while mass balance calculations are detailed in supplementary material (section S1). Considering the H_2S stripping in the UASB, the sulfur imbalance along the operation oscillated between −10% and +20%. The sulfur imbalance was attributed to the production of undetected sulfur species in the rector. As reported by Mora et al. (2020b), the sulfur imbalance was not caused by the metallic sulfide precipitating

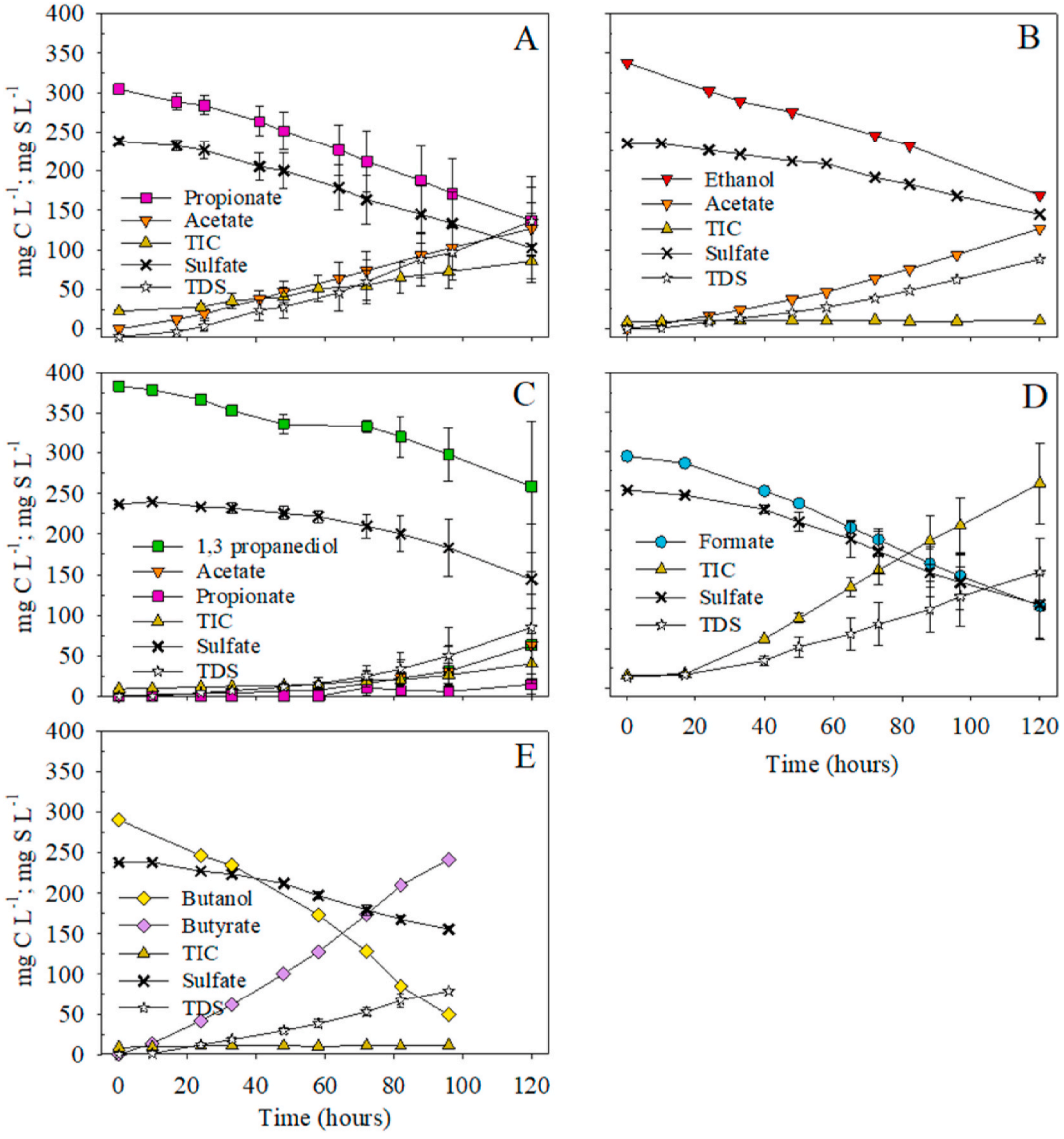

Fig. 2. Carbon and sulfate conversions in the experimental bottles feeding sulfate plus propionate (A), ethanol (B), 1,3-propanediol (C), formate (D) and butanol (E). Error bars represent standard deviations of duplicate tests. TIC represents total inorganic carbon.

but due to the production of organic sulfur compounds as intermediate compounds of the biodegradation. The negative imbalance was attributed to the standard deviation in the measurement of TDS (8%). Considering CH_4 and CO_2 stripping on the gas phase of UASB, the carbon imbalance was mainly between −5% and 30%, which is in concordance with the sulfur imbalance. The positive imbalance was attributed to the growth of biomass in the reactor according to previous references (Fernández-Palacios et al., 2019).

3.2. Sulfate reduction with single organic compounds

During the sulfate reduction with glycerol, it was found that SRB metabolized simple organic carbon intermediate products (Fig. 1C and E). Combined with the description of the fermentation mechanism of glycerol described by Viana et al. (2012), the intermediate products observed in Fig. 1 were used as individual carbon sources to further study the mechanism and rates of the sulfate reduction process.

When no sulfate was added, the carbon content remained constant when sludge was fed with propionate (Fig. S4A), ethanol (Fig. S4B), 1,3-propanediol (Fig. S4C) and butanol (Fig. S4E). This indicates that granular sludge was not capable of using those carbon sources without sulfate. However, in the test that used formate as the carbon source (Fig. S4D), and after 40 h of lag time, this compound was consumed at a rate of 2.8 ± 0.8 mg C g VSS^{-1} h^{-1} while inorganic carbon was accumulated at a rate of 3.6 ± 1.3 mg C g VSS^{-1} h^{-1}. Fig. 2 shows the profiles of C and S species when single electron donors produced during glycerol fermentation were fed in batch tests to the UASB sludge. As can be observed, propionate (Fig. 2A), ethanol (Figs. 2B), 1,3-propanediol (Fig. 2C), formate (Fig. 2D) and butanol (Fig. 2E) were consumed in the sulfate-added tests. Coupled to sulfate reduction, propionate was degraded to acetate and inorganic carbon according to Eq. (8) (Table 1), ethanol was degraded to acetate according to Eq. (9) (Table 1), formate was degraded to inorganic carbon, and butanol was oxidized to butyrate. When 1,3-propanediol was added, sulfate was reduced to sulfide and the consumption of 1,3-propanediol was accompanied by the accumulation of acetate, propionate and inorganic carbon (Fig. 2C). Despite the VFA production, the buffering capacity of the mineral medium maintained the pH above 7.5.

3.2.1. Sulfate reduction with propionate

According to literature, there are two main pathways described for the sulfate reduction process using propionate as electron donor. One is acetogenesis, in which propionate is degraded into acetate and hydrogen by acetogens (Li et al., 2017), and then sulfate is reduced both by hydrogenotrophic and acetotrophic SRB (Muyzer and Stams, 2008). The other is the propionate oxidation pathway by propionate-degrading sulfate reducers, which directly uses propionate as electron donor to reduce sulfate. The oxidation pathway includes partial oxidation of propionate to form acetate and bicarbonate and complete oxidation of propionate to bicarbonate (Luis, 2018). In the absence of sulfate, acetate and bicarbonate were not produced in this work, showing that there was no acetogenesis from propionate. However, propionate was incompletely oxidized to acetate in the presence of sulfate while acetate could not be further fermented to produce methane nor used to reduce sulfate, as shown in supplementary material (Fig. S5). Although many studies have shown that acetate can be oxidized to CO_2 by SRB, including *Desulforhabdus amnigenus*, *Desulfobacca acetoxidans*, *Desulfarculus baarsii* and *Desulfatitalea* (Higashioka et al., 2013; Oude Elferink et al., 1998; Widdel and Pfennig, 1977), they were not present in this work as confirmed by 16S rRNA sequence analysis. In previous works in the same UASB reactor fed with crude glycerol as studied herein, it was reported that acetate accumulated in the long-term after increasing the sulfate loading rate or after a 24 h pH shock (Fernández-Palacios et al., 2019; Mora et al., 2020b), since methanogens were probably the only microorganisms that consumed acetate. After methanogens wash out, acetate cannot be further oxidized due to the lack of acetotrophic SRB. H_2S inhibition could be another reason that lead to incomplete oxidizing SRB to dominate among SRB species. Maillacheruvu and Parkin (1996) reported that acetotrophic SRB and propionate fermenters were more sensitive to sulfide toxicity compared to hydrogenotrophic SRB and incomplete propionate-utilizing SRB. Thus, the mechanism of sulfate reduction using propionate was considered to be partial oxidation, represented by Eq. (8). This was confirmed by comparing the stoichiometric rates and the experimental rates (Table S1). Their calculation process is shown in supplementary material (section S3.1).

3.2.2. Sulfate reduction with ethanol and 1,3-propanediol

In the control bottles fed with ethanol as carbon source, the ethanol concentration remained constant and no other products were produced (Fig. S4B). However, in the presence of sulfate, sulfate was reduced to sulfide while ethanol was converted to acetate (Fig. 2B). When sulfidogenesis dominated in a long-term UASB treatment with sulfate-rich wastewater, SRB oxidized ethanol into acetate for sulfidogenesis according to Eq. (9) (Wu et al., 2018). The experimental results of Fig. 2B are well-explained through Eq. (9), which was confirmed by the stoichiometric calculation in Table S1.

Similarly, results showed that 1,3-propanediol was not degraded to VFAs or inorganic carbon in the absence of sulfate (Fig. S4C). However, in the presence of sulfate, 1,3-propanediol was oxidized to acetate, propionate, and bicarbonate (Fig. 2C). Qatibi et al. (1991b) reported that 1,3-propanediol was oxidized to acetate and bicarbonate according to Eq. (10) during sulfate reduction (Table 1). They found that 3-hydroxypropionate, which was further oxidized to acetate, was the intermediate of the degradation of 1,3-propanediol by *Desulfovibrio alcoholovorans*. Interestingly, Liu and Liu (2016) reported that propionate was produced from 3-hydroxypropionate by *Metallosphaera sedula* through the autotrophic carbon dioxide assimilation cycle through 3-hydroxypropionyl-CoA synthesis, 3-hydroxypropionyl-CoA dehydration, acryloyl-CoA reduction, and CoA hydrolysis (Fig. 3). Then, 1,3-propanediol ($C_3H_8O_2$) oxidation to propionate ($C_3H_5O_2^-$) was lumped into Eq. (11) in this work.

$$C_3H_8O_2 + SO_4^{2-} + 3NADH + 3H^+ \rightarrow C_3H_5O_2^- + HS^- + 4H_2O + 3NAD^+ \quad (11)$$

Consequently, both Eqs. (10) and (11) were used in the work herein to describe the mechanism for the oxidation of 1,3-propanediol coupled to the sulfate reduction following the pathway described in Fig. 3.

3.2.3. Sulfate reduction with formate and butanol

Despite some extra inorganic carbon was produced based on the formate consumed, which was attributed to some remaining COD in the granules before the test, formate was oxidized to produce inorganic carbon in the absence of sulfate (Fig. S4D). In addition, 2.6 mg L^{-1} of H_2 were detected in the headspace of serum bottles at the end of the test. Thus, formate was converted into CO_2 and H_2 according to Eq. (5) (Table 1). In the presence of sulfate, sulfate was reduced to TDS, while formate was oxidized to carbonate (Fig. 2D). Similarly, 7.6 mg L^{-1} of H_2 were also detected after cultivation. It has been reported that formate is rapidly oxidized into CO_2 and H_2; then, hydrogen can be used as electron donor for further sulfate reduction by hydrogenotrophic SRB (Bijmans et al., 2008) according to Eq. (6) (Table 1). It is worth mentioning that formate can also be used as electron donor to directly reduce sulfate by SRB (De Smul and Verstraete, 1999; Liamleam and Annachhatre, 2007). If all formate was oxidized and the H_2 produced was used for sulfate reduction only according to Eqs. (5) and (6), respectively, the corresponding SRR calculated by the stoichiometric equations would be 3.8 ± 1 mg S g VSS^{-1} h^{-1}. Instead, the actual experimental SRR was 5.2 ± 0.9 mg S g VSS^{-1} h^{-1}. Consequently, formate was converted into CO_2 and H_2 as well as used as electron donor for sulfate reduction (Eq. (7)). In addition, hydrogen was used by SRB as electron donor for sulfate reduction as represented by Eq. (6).

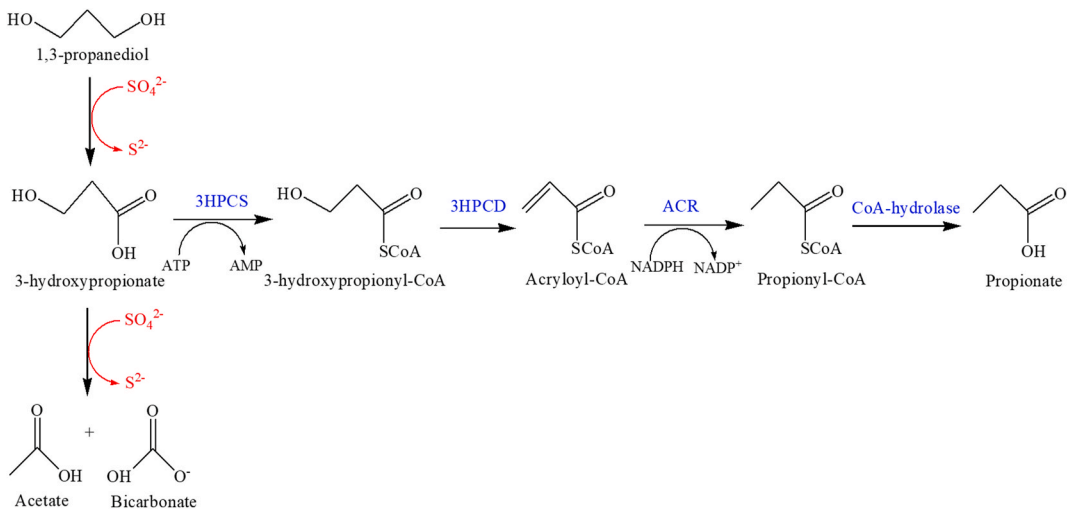

Fig. 3. The metabolic pathway for the conversion of 1,3-propanediol into propionate, acetate and bicarbonate proposed in this work based on the mechanisms described in Qatibi et al. (1991b) (vertical pathway) and Liu and Liu (2016) (horizontal pathway). 3HPCS, 3-hydroxypropionyl-CoA synthetase; 3HPCD, 3-hydroxypropionyl-CoA dehydratase; ACR, acryloyl-CoA reductase.

Despite butanol was not found in the batch test with glycerol (Fig. 1A and C), it has been reported as an electron donor for SRB to reduce sulfate. Particularly, *Desulfovibrio* spp. are capable to incompletely oxidize butanol to succinate coupled to sulfate reduction (Dowling et al., 1992). Sarti and Zaiat (2011) found that butanol was converted to acetic acid by incompletely oxidizing SRB in an anaerobic sequential batch reactor. Due to the significant presence of *Desulfovibrio*, it was decided to test butanol with the granular biomass from the UASB reactor. In the work herein, butanol was not degraded in the absence of sulfate (Fig. S4E). When sulfate was added, all butanol was converted to butyrate while no acetate, other VFA nor alcohols were produced (Fig. 2E). This observation has not been reported previously in literature and the specific strain of SRB able to oxidize butanol to butyrate needs to be further confirmed. Although sulfidogenic granular sludge can oxidize butanol to butyrate, no other organic compounds and inorganic carbon were produced during the oxidation of butanol to butyrate, which means that SRB could not further use butyrate formed to reduce sulfate. As observed in Fig. 1C and D, glycerol was not converted into butanol or butyrate in the sulfate reduction process. Similarly, no VFAs were produced when only 2,3-butanediol was added (Fig. S6A). Similar results were obtained adding sulfate (Fig. S6B), thus indicating that there were no butanediol-degrading anaerobes in this work.

3.2.4. *The mechanism of biodegradation of glycerol and sulfate reduction*

Based on the batch tests results, a mechanism for sulfate reduction using glycerol as electron donor was proposed in this work (Fig. 4). From

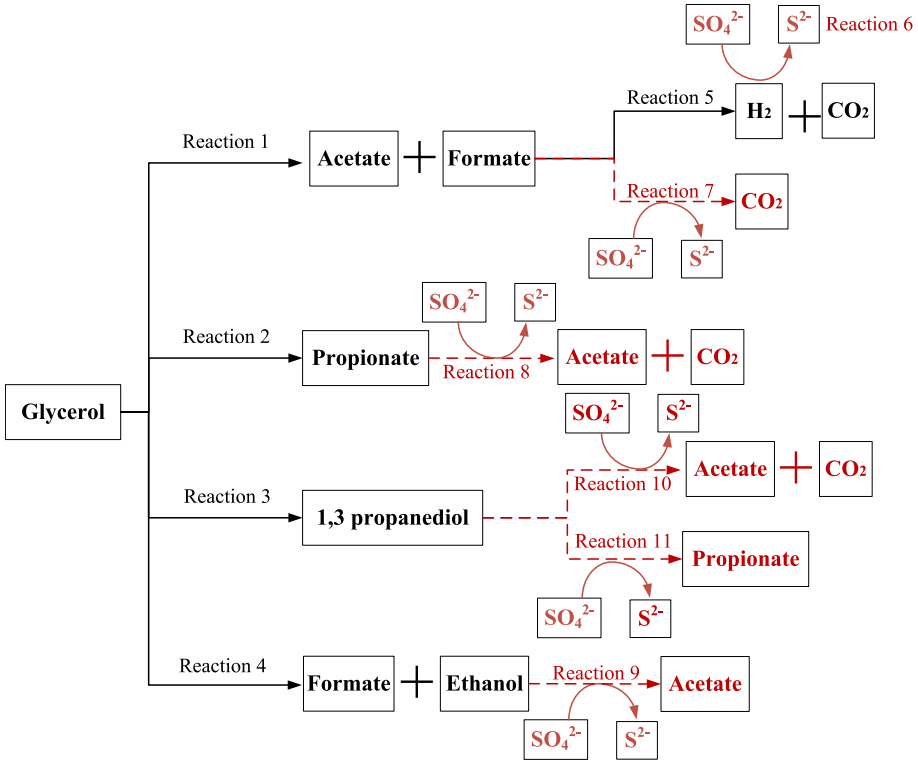

Fig. 4. The main mechanism of biodegradation of glycerol and sulfate reduction without methane production. The repeated degradation and sulfate reduction pathways are not listed, such as propionate produced by reaction 11 and formate produced by reaction 4. Black reactions and continuous lines represent the fermentation process, while red reactions and discontinuous lines represent the sulfidogenic process. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the sulfate reduction process performed by each single electron donor under non-methanogenic conditions, it was concluded that the granular sludge taken from the UASB operated under long-term sulfidogenic conditions while fed with crude glycerol was 1) able to ferment glycerol to acetate, formate, propionate, ethanol and 1,3-propanediol; 2) able to use H_2, formate, propionate, ethanol and 1,3-propanediol for sulfate reduction; 3) able to oxidize formate to CO_2 and H_2; and 4) there were no acetogens that used propionate, ethanol nor 1,3-propanediol directly. Despite other intermediates not considered in this mechanism play a role in the detailed metabolic pathways of the microbial cultures grown in the UASB, the approach proposed herein provides a simplified view of the granular sludge activity through the main VFA and alcohols involved in glycerol fermentation. Such an approach contributes to expand the knowledge of sulfate reducing bioreactors and clarifies the mechanism of sulfate reduction through intermediate products and their contribution to the sulfate reduction process.

3.3. Contribution of different electron donors to sulfate reduction

Table 2 shows the specific rates of different electron donors consumption and sulfate reduction in batch activity tests. Specific rates were obtained by dividing volumetric rates by VSS, while volumetric rates were calculated based on data from Fig. S4 and Fig. 2 as described in section 2.3. In the present work, SRRs of tests performed with single electron donors were ranked as follows: ethanol > formate > butanol >1,3-propanediol > propionate. The specific SRRs using ethanol and formate were 5.8 and 5.2 mg S g VSS^{-1} h^{-1}, respectively. Thus, the granular sludge cultivated under sulfidogenic anaerobic conditions of continuous feeding with crude glycerol promoted formate-utilizing and ethanol-utilizing SRB species. SRB tend to use low molecular weight (MW) organic compounds in the cultivation of organic matrix mixtures (Neculita et al., 2007), which also has the benefit of improving sulfate reduction efficiency (Zhao et al., 2010). As can be observed from Table 2, the SRR obtained using formate and ethanol (compounds containing two carbons or less) was 39% higher than that obtained with compounds containing three carbons or more on average (propionate, 1, 3-propanediol and butanol), which is in agreement with Neculita et al. (2007). Vallero et al. (2004a) reported a SRR of 9.5 mg S g VSS^{-1} h^{-1} in a methanogenic sludge fed with formate while Wu et al. (2018) reported a SRR of 66.2 mg S g VSS^{-1} h^{-1} using ethanol as sole carbon source, the later reported in a sulfidogenic dominant stage. The difference of SRRs between our work and previous studies was related to the development of microbial populations acclimated to a specific carbon source providing thus a larger affinity for such low-MW electron donors.

Table 3 shows the contribution of each electron donor to the overall SRR from the batch tests in which pure glycerol and sulfate were fed. The calculation process is shown in supplementary material (section S3.2). From 33 to 96 h, sulfate was mainly reduced using ethanol and 1,3-propanediol as electron donors. The overall contribution of these two compounds to the total sulfate reduction along the test with glycerol plus sulfate accounted for 78.6%. However, within 33–48 h, SRB mainly used ethanol to reduce sulfate rather than formate or 1,3-propanediol since the concentration of ethanol fermented through glycerol was 2.4 times that of formate after 33 h (Fig. 1C and D). After ethanol was consumed in the sulfate reduction process (48 h), 1,3-propanediol became the main electron donor for the reduction of sulfate. Once glycerol was completely degraded after 33 h (Fig. 1C), the production of 1,3- propanediol was 4.4 times that of formate. According to Table 2, the specific SRR for formate was higher than that of 1,3-propanediol. However, formate was not a key contributor to the overall sulfate reduction, probably due to its low-production from glycerol fermentation. The predominance of ethanol and 1,3-propanediol as main electron donors for sulfate reduction is consistent with previous works in which glycerol fermentation in anaerobic digestion lead also to a major production of both compounds (Metsoviti et al., 2012; Rossi et al., 2012; Wu et al., 2011). However, it is worth mentioning that the contribution of

Table 2
Specific rates obtained from the sulfidogenic anaerobic digestion in batch activity tests.

Electron donor	Initial carbon concentration (mg C g L^{-1})	TOC/ $S\text{-}SO_4^{2-}$	T (°C)	Initial pH	Final pH	Specific substrate consumption rate (mg C g VSS^{-1} h^{-1})	Specific sulfate reduction rate (mg S g VSS^{-1} h^{-1})	Reference
Formate	1500	0.37	65	7.8	N.A.	92.8 ± 6.5	9.5 ± 1.9 [a]	(Vallero et al., 2004a, b)
Ethanol	750	0.27	35	N.A.	N.A.	N.A.	66.2 [b]	Wu et al. (2018)
Formate	372	1.52	35	7.9	7.8	6.9 ± 1.5	5.2 ± 0.9 [c]	This study
Propionate	342	1.41	35	7.9	7.5	4.4 ± 0.5	3.7 ± 0.8 [c]	This study
Ethanol	318	1.35	35	8.4	7.6	9.3 ± 5.1	5.8 ± 1.5 [c]	This study
1,3-propanediol	339	1.43	35	8.4	7.8	4.7 ± 0.5	3.9 ± 1.1 [c]	This study
Butanol	334	1.40	35	8.4	7.5	11.6 ± 4.4	4.3 ± 1.6 [c]	This study

Note: N.A. not applicable.
[a] The specific rate was calculated by taking out the granular sludge from the UASB after 55 days of operation.
[b] The specific rate was calculated by taking out the granular sludge from the UASB after 330 days of operation.
[c] The specific rate was calculated by taking out the granular sludge from the UASB after 431 days of operation.

Table 3
Stoichiometric specific SRRs according to Eqs. (1)–(11), proposed mechanism and percentage contribution of each electron donor to the observed SRR of granular sludge fed with pure glycerol.

Time (h)	Formate and H_2 mg S g VSS^{-1} h^{-1} (%)	Propionate mg S g VSS^{-1} h^{-1} (%)	1,3-propanediol mg S g VSS^{-1} h^{-1} (%)	Ethanol mg S g VSS^{-1} h^{-1} (%)	Observed SRR mg S g VSS^{-1} h^{-1} (%)
33–48	2.2 (20.9%)	0	2.6 (25.3%)	5.9 (57.0%)	10.4
48–72	0.4 ± 0.57 (6.0 ± 8.7%)	1.1 ± 2.2 (16.2 ± 33.2%)	5.2 ± 1.2 (77.7 ± 27.1%)	0	6.7 ± 1.8
72–96	0	0.1 ± 1.1 (3.3 ± 31.9%)	3.3 ± 0.5 (88.8 ± 20.5%)	0	3.7 ± 0.6

the different electron donors depends on several factors such as physiological differences among microbial cultures, the type of fermentation, substrate concentration and possible inhibitions (Biebl et al., 1999). Wu et al. (2011) reported that an excess amount of glycerol may cause a metabolic product shift from ethanol to 1,3-propanediol. Therefore, conditions can be accommodated to create an environment that is more conducive to the growth of SRB, such as the production of simpler carbon sources that SRB would use preferentially over complex ones.

4. Conclusions

The research in this work permitted to establish a simplified mechanism of sulfate reduction by granular sludge from a long-term operated sulfidogenic UASB using glycerol as electron donor. It also provides the experimental rates and stoichiometric equations considering the contribution of the intermediate products produced by the fermentation of glycerol previous to the sulfate reduction step. Under non-methanogenic but sulfidogenic conditions, SRB did not directly used glycerol to reduce sulfate. Glycerol was firstly fermented by the granular sludge to form simpler intermediates such as H_2, formate, propionate, ethanol, 1,3-propanediol, and then SRB reduced sulfate with such intermediate products. The sulfate reduction process mainly used 1,3-propanediol and ethanol as electron donors, which were produced through glycerol fermentation. Butanol was not an intermediate product of glycerol fermentation, but it was capable to be oxidized to butyrate in the presence of sulfate. SRB performing an incomplete oxidation of the electron donors dominated in the granular sludge from the sulfidogenic UASB reactor. SRB preferred to use simple intermediate products containing less than 2 carbons as electron donors, except in the case of acetate that was not found to be used for sulfate reduction. *Desulfobulbus* and *Desulfovibrio* were found as the main sulfate reducing genus, accounting for 22.4% of the total amount of sequences detected under sulfidogenic conditions.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.131649.

Credit author statement

Xudong Zhou: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization Eva Fernández-Palacios: Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. Antoni D. Dorado: Writing – review & editing, Visualization, Supervision Xavier Gamisans: Resources, Writing – review & editing, Supervision, Funding acquisition. David Gabriel: Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition

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