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Bioelectrochemical treatment of acid mine drainage: Microbiome synergy influences sulfidogenesis and acetogenesis

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ABSTRACT

Bioelectrochemical systems (BES) are emerging as potential technologies that can remediate acid mine drainage (AMD) by cathodic reduction of sulfates to metal sulfides. This study evaluated bioelectrochemical remediation of sulfate rich AMD at two applied cathode potentials; BES-1: −1.0 V and BES-2: −0.8 V. Sulfate reducing bacteria were selectively enriched to be used as biocatalyst in BES. Initially, lactate was fed as carbon source and switched to chemolithoautotrophy with only CO_2-fed conditions. Both BESs were operated at 3±0.2 g/l of sulfate with synthetic AMD (SAMD) fed first, and gradually changed to 50% AMD from mining site with 50% SAMD. Sulfate reduction was relatively higher with BES-1: 82% than BES-2: 76% coupled with sulfidogenesis. Interestingly, acetogenesis (BES-1: 2.12±0.2 g/l, BES-2: 1.9±0.2 g/l) was also noticed with high reduction currents (BES-1&2: >-70 mA). Microbiome community analysis revealed the dominant presence of sulfate reducers, acetogens, syntrophic bacteria and *Methanobacterium*, probing microbial synergy aiding sulfate reduction. An added advantage was the iron-sulfide (FeS) particles formation on cathode, which might have contributed to increased reduction currents. This study reveals insights into microbial synergy for autotrophic sulfate reduction within mixed microbiome communities along with the impact of FeS particles as conducive facilitator for electron transfer in BES, thereby enhancing electrosynthetic acetate production.

1. Introduction

Acid mine drainage (AMD), an unavoidable by-product of the mining and mineral industry which is characterized by low pH, high concentrations of sulfate, metals and other elements, is a widespread environmental concern [1]. Currently, mining industry is under unprecedented pressure as the discharge of metal laden waste and high sulfate bearing minerals into the water bodies and ecosystems via AMD pose a significant threat [2]. This requires a critical attention for AMD remediation, prior to its discharge into the environment [3]. Conventional methods of AMD remediation are mostly chemical based techniques that requires alkaline chemicals to neutralize the high acidic content of AMD, which subsequently results in the generation of recalcitrant sludge and gypsum as sulfate precipitate. The unsustainability and energy intensity of these processes, underscore for alternative, eco-friendly, sustainable, and less energy-intensive solutions for remediating AMD and sulfate rich wastewaters [4]. Biological sulfate reduction is a promising approach that is widely studied for the treatment of sulfate-laden wastewaters, mostly requiring organic carbon as an electron donor [5]. Sulfate reducing bacteria (SRB) are promising candidates that perform sulfate reduction coupled with sulfide generation [6,7]. In general, SRB are mostly heterotrophic relying on organic carbon as electron donor for carrying out sulfate reduction reactions, while it is also possible via hydrogenotrophic SRB. However, biological sulfate reduction by SRB depends on organic carbon, either provided externally or generated by acetate consumption via acetogenesis from CO_2 by acetogenic bacteria that co-exist in the microbiome.

Although biological sulfate reduction by SRB occurs at good rates, addition of external organic carbon limits their practical application, as AMD is typically lacking organics [2]. External supplementation of electron donors aggravates the cost of overall process, thereby prohibiting their application at a real scale. To address this issue, there is an urgent need for a sustainable process that can achieve autotrophic sulfate reduction and AMD remediation at a large scale. In view of this,

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bioelectrochemical systems (BES) are a rapid and sustainable option being studied for various applications, including sulfate reduction at high rates surpassing the limitations of conventional treatment methods that results in recalcitrant sludge generation associated with the use of high costs of chemical usage [6,8]. Major advantage associated with BES is facilitation of autotrophic sulfate reduction by SRB with the electrode serving as electron donor and without supplementing any organic carbon externally [9,10]. Although it is believed that electro autotrophic sulfate reduction is attained in BES with electrode serving as sole electron donor, there are several possible mechanisms that could occur towards attaining sulfate reduction without external addition of organics. Contrary to the pure culture studies with potential SRB as biocatalyst, it is challenging to determine the mode of sulfate reduction in mixed microbiome communities. The process could occur either via hydrogenotrophic SRB or heterotrophic SRB, with the latter relying on acetate obtained via acetogenesis by homoacetogens. However, this seems to be advantageous if two or more processes occur through the multi-species bacterial synergy, favoring sulfate reduction. While BES has been studied for the treatment of high strength sulfate containing wastewaters, there are also studies dealing with BES for AMD treatment [2,6,8, 11–15]. Sulfate reduction coupled with sulfidogenesis is either in the form of dissolved sulfide (HS^-) or gaseous sulfide (H_2S). Hydrogen sulfide (H_2S) in gaseous form is toxic to the co-existing microbial community and in addition, its accumulation in higher quantities could result in reduced process efficiency [14]. Nevertheless, treatment of AMD containing metals along with high concentration of sulfates would result in sulfide (HS^- (l) or H_2S (g)) formation that would ultimately form metal sulfides (MS^-) which can be precipitated and recovered for potential applications. Metal sulfide particles could be nano, micro, or macro sized, and have several applications and economical value if separated and recovered [16]. Some of the metal sulfide particles have conducting capabilities that would benefit the current processes occurring in BES by aiding in electron transfer. In BES, electron transfer from or towards electrode is an important phenomenon which regulates the process efficiency [17]. Addition of conductive particles or mediators as electron shuttles that augment electron transfer has been widely studied in microbial fuel cell (MFC), and few electrogenic bacteria are known to use electron shuttles for rapid electron transfer [18]. In AMD, presence of metals which is regarded as a problem, can be reconsidered as an advantage in BES, as the metal sulfides tend to form spontaneously in the presence of sulfides, and are ought to have an advantage of electron conducting properties. This could also potentially act as electron conduits between two or more bacteria and between electrode and bacteria, thus minimizing electron losses and aiding in enhanced electron transfer rates towards benefitting the process [19–21].

Another important point to be noted is the synergy among different bacteria that thrive and co-exist along with SRB in sulfate rich wastewaters and AMD [2]. It is imperative to know that several groups of bacteria, such as homoacetogens, methanogens, hydrogenotrophs, syntrophic acetate oxidizing bacteria, chemolithoautotrophs and methanogenic archaea exist together with SRB, which can function in synergy with each other or independently. Mixed microbial communities enriched with SRB, do constitute the aforementioned groups of organisms, and their interplay depends on the availability of electron donor, sulfate concentration and the operating microenvironment as discussed in several studies [2,6,13,14]. While there is currently limited knowledge regarding the synergy in SRB enriched bioreactors, the interplay of several conditions, such as autotrophic sulfate reduction in BES, the impact of metal sulfides, co-occurrence of acetate and hydrogen production, synergy among different microbial groups despite the low abundance of known potential sulfate reducers at the cathodic environment, are important to understand for grasping the long-term functionality of microbiome.

Considering the above-mentioned gaps, this study is aimed at studying autotrophic sulfate reduction in BES, using enriched SRB from mixed anaerobic sludge. Autotrophic sulfate reduction and sulfidogenesis were studied at two different applied voltages in two BES operated independently. This study is also aimed at developing a microbial community to treat AMD wastewaters by prior adaptation to sulfate rich environments, that could potentially be enriched as a stable microbial community. The impact of metal sulfide particles formation on the electron transfer and reduction currents was noted and discussed accordingly. Microbiome community structure was analyzed in detail and the synergy between diverse microbial groups for augmenting sulfate reduction and simultaneous acetogenesis were discussed. This study will unleash new insights on understanding autotrophic sulfate reduction in enriched microbiome even in conditions of low abundance of potential sulfate reducers in BES.

2. Materials and methods

2.1. Acid mine drainage

In addition to AMD from mining site, supplied by Boliden Mineral AB, simulated wastewater that mimics AMD composition with high concentrations of iron and sulfate ($FeSO_4.7\ H_2O$) was prepared and used. Simulated AMD wastewater (SAMD) composed of $CoCl_2$: 25 mg/l, $ZnCl_2$: 11.5 mg/l, $CuCl_2$: 10.5 mg/l, $CaCl_2$: 5 mg/l, $MnCl_2$: 15 mg/l, KH_2PO_4: 0.25 g/l, K_2HPO_4: 0.25 g/l, NH_4Cl: 0.5 g/l, $MgCl_2$: 0.3 g/l, $NiSO_4$: 0.16 g/l, $FeCl_3$: 0.03 g/l, $FeSO_4.7\ H_2O$ accounting to 3±0.2 g/l sulfate was used as media for experimental operations along with yeast extract (0.5 g/l) as nitrogen source for bacteria growth. Subsequent operations were carried out with 50% SAMD and 50% AMD from mining site (labeled as BN-22 as supplied by Boliden Mineral AB). The AMD from mining site (AMD_BN22) that consisted of various metals/elements were characterized as follows ((μg/l) Al: 146, As: <0.5, Ba: 22, Cd: 21.5, Cr: <0.5, Co: 17.4, Cu: 39.9, Fe: 4.66, Pb: <0.2, Mn: 1630, Mo: 1.61, Ni: 29.2, Zn: 6240, Sn: 2.13, Sr: 508, U: 0.121, SO_4^{2-}: 852 mg/l, K: 17 mg/l, Na: 56.1 mg/l, Si: 9.15 mg/l, S: 262 mg/l). By dosing a few ml of stock $FeSO_4$ solution, the final sulfate concentration of 3±0.2 g/l was maintained in the mixed AMD sample.

2.2. Biocatalyst

As AMD is rich in sulfur/sulfate bearing minerals/metals, sulfate reducing bacteria (SRB) is an efficient biocatalyst that can be used for facilitating sulfate reduction to sulfide. A selective enrichment procedure was adopted for the growth of sulfate reducing bacteria to be used as biocatalyst for performing AMD remediation. Anaerobic mixed culture originally acquired from a biogas plant in Luleå, Sweden was taken and subjected to heat pretreatment(treatment at 80–90°C for 1 hour) to be used as parent inoculum for the enrichment of SRB. [22]. The sludge sample from biogas plant was first heat-treated and the treated sample was inoculated into the degassed enrichment media (DSM 63 media) in several serum bottles. The original sludge sample was not filtered/degassed, and only the cultivation media used for growing SRB was degassed. For the enrichment stage, the media contained a carbon source (either glucose or fructose or lactate), Na_2SO_4 and yeast extract. SRB were selectively enriched heterotrophically in serial transfer of cultivation in several vials. The cultures grown in the last culture transfer were used to inoculate the cathode side of BES reactors. Later in the BES operation phase, organic carbon sources were omitted and CO_2 was provided as a carbon source to selectively grow the autotrophic SRB from the mixed consortia. During the operation in BES reactor with CO_2 feed, switch from heterotrophic to autotrophic mode of sulfate reduction occurred. 15 ml of parent inoculum was taken and suspended in 35 ml of enrichment medium (DSM 63) in serum bottles with a total volume of 150 ml. Three different carbon sources such as glucose, fructose, and lactate with an initial concentration of 1.5 g/l were chosen and a standard sulfate concentration of 3 g/l of sodium sulfate (Na_2SO_4) was added to the enrichment cultures and the pH was adjusted to 6. Successful enrichment was assessed based on the reduction in carbon and sulfate

concentration. All the enrichments were carried out in triplicates for each carbon source. The enrichments were designated as SRB-G, SRB-F and SRB-L, fed with glucose, fructose and lactate respectively. Successfully enriched bacteria that performed efficient sulfate reduction associated with carbon utilization was chosen further to be used in two dual chambered BES for sulfate reduction.

2.3. Bioelectrochemical systems

Two double chambered H-type glass reactors, each with a working volume of 200 ml, were used for the designated BES-1 and BES-2. Anode chambers were operated with an abiotic buffer solution (0.5 g/L NH_4Cl, 0.25 g/L KH_2PO_4, 0.25 g/L K_2HPO_4, 0.3 g/L $MgCl_2$, 25 mg/L $CoCl_2$, 11.5 mg/L $ZnCl_2$, 10.5 mg/L $CuCl_2$, 5 mg/L $CaCl_2$, 15 mg/L $MnCl_2$, 16 mg/L $NiSO_4$, and 25 mg/L $FeCl_3$), while cathode chambers consisted of SAMD followed by mixture of SAMD and AMD from mine site (50:50) as media, enriched with SRB culture as biocatalyst. A pretreated proton exchange membrane (PEM) Nafion 117 (Ion Power GmbH, München-Flughafen, Germany) separated the anode and cathode chambers which also facilitated proton transfer from anode to cathode. For the electrodes, anode chambers were operated with graphite rods (Schunk Carbon Technology AB, Sweden), with the dimensions of 20 mm diameter and 70 mm length. Stainless steel (SS) rod of 2 mm diameter held the graphite rod hanging from the top stopper in the reactor and was also used as a current collector. Heat-shrink insulating tube was used to cover the SS rod and to avoid its contact with the electrolyte as previously described [23]. About 50 mm of the anode was submerged in anolyte. Each cathode chamber accommodated a carbon cloth as a cathode with the dimensions 6.5 cm length and 2.5 cm width. Carbon cloths were connected to the working lead of a potentiostat using a titanium wire as current collectors. An Ag/AgCl reference electrode ET072–1 (eDAQ, Australia) was used in the cathode chamber to poise the potential of working electrode (cathode).

2.4. Operation

BES reactors were operated in batch mode under mesophilic conditions (temperature-controlled plexiglass box at 35°C) by feeding SAMD at the cathode chamber. A potential of −1.0 V vs Ag/AgCl was applied on BES-1, while BES-2 was operated with an applied potential of −0.8 V vs Ag/AgCl using a Multi Emstat3+ potentiostat (Palmsens, The Netherlands). Both the BES were operated by setting initial pH to 6.5. Enriched SRB culture, inoculated as biocatalyst, was used at the cathode to reduce sulfate into sulfide by utilizing the electrons driven from the anode via hosting oxygen evolution or chlorine evolution reaction with the aid of voltage. Maximum reduction in sulfate concentration (measured as per the analysis mentioned in Section 2.5) was used as an indicator for initiating a new batch run for BES operation. Magnetic beads were placed inside the cathode chamber to ensure proper mixing and to avoid a concentration gradient. Besides, CO_2 gas recirculation was arranged for each BES at the cathode chamber with the aid of a gas bag holding ~3 l of CO_2. Continuous CO_2 recirculation from the gas bag to the reactor was intended to promote anaerobic and chemolithotrophic conditions. After operating BES with SAMD, a 50/50 (v/v) mixture of AMD from mine site and SAMD was fed to the reactors.

2.5. Analysis

BES reactors were assessed for their biochemical and bioelectrochemical performances. Biochemical parameters, such as sulfate reduction and sulfide formation, were analyzed periodically using the test kits obtained from Merck Spectroquant® Supelco® (100–1000 mg/L range for sulfate and 0.020–1.50 mg/L range for sulfide). Quantitative and qualitative estimation of organic metabolites formed during BES operation and carbon reduction during enrichment phase were analyzed by high performance liquid chromatography (HPLC). HPLC was carried on a PerkinElmer apparatus equipped with a Bio-Rad Aminex HPX-87 H column (300 m 37.8 mm), a Flexar LC pump and a PerkinElmer-200 refractive index detector (RID). 5 mM H_2SO_4 was used as mobile phase eluted at 0.6 ml/min flow rate. The temperature of column was maintained at 65 °C. Change in pH was measured using pH meter [pHenomenal pH 1100 L pH meter, (VWR, Germany)]. Bioelectrochemical performance of BES, in terms of reductive catalytic currents from chronoamperometric measurements as well as cyclic voltammograms, were recorded and analyzed in the specified voltage ranges using potentiostat-galvanostat system from MultiEmStat+ potentiostat (Palmsens, NL) with the aid of channels in three-electrode setup. Decrease in sulfate concentration and reduction currents were used as indicators for batch change. Structural and elemental characterization of cathode biofilm and suspended microbial biomass from BES operation were performed using scanning electron microscopy (SEM, FEI Magellan 400 field emission XHR-SEM) and electron dispersive spectroscopy (EDS, X-Max 80 mm^2 EDS spectrometer, Oxford Instruments) with Aztec version 3.3 (oxford instruments) as software. Liquid samples with suspended biomass from the BES reactor were centrifuged and the supernatant was removed. The pellet biomass samples were frozen at −20 ℃ and then freeze-dried for two days. The dried samples were used for SEM imaging. In case of biofilm imaging on cathode, a small piece of electrode sample was cut from the BES cathode and washed with phosphate buffer solution to remove the loose biomass. The sample was then frozen at −20 ℃ and then subjected to freeze drying for two days. The dried samples were then subjected to SEM imaging. The samples were fixed with 2.5% glutaraldehyde and vacuumed dried first, which were subsequently placed on conductive carbon tapes prior to the analysis. SEM images were captured at a voltage of 3 kV and a beam current of 6.3 pA, while in the case of EDS analysis, accelerating voltage of 20 kV and a beam current of 0.2 nA were employed with a working distance of 5 mm.

2.6. Microbial inventory

Samples were collected from BES reactors for analyzing the microbial community as described [24]. Microbial DNA was extracted using the FastDNA soil spin kit (MP Biomedicals, USA). The purity and size of subset of DNA extracts were confirmed and quantified as discussed in [24]. To create tailed primers encoding the sequences GTGYCAGCMGCCGCGGTAA (515FB) and GGACTACNVGGGTWTCTAAT (806RB) that target the Bacterial/Archea 16 S rRNA gene variable region 4 (abV4-C), an Illumina procedure was modified. After PCR amplification, DNA was eluted and Qubit ds DNA hs Assay was used to measure the concentration. Equimolar concentrations of purified DNA sequencing libraries were taken and paired-end sequencing (2–3, 300 bp) was carried out on an Illumina MiSeq platform using the MiSeq Reagent Kit v3 and following normal procedure. Details regarding the softwares and bioinformatics processing is described in Sarkar et al. [24].

3. Results and discussion

3.1. Enrichment of sulfate reducing bacteria

A selective enrichment procedure as described in Section 2.2 was adopted for the growth of sulfate reducing bacteria to be used as biocatalyst for performing AMD remediation. Among the three carbon substrates tested, sulfate reduction was higher in SRB enrichments fed with lactate, with a sulfate reduction of 92.7%, while 78% and 72% were obtained for SRB-F and SRB-G respectively (Fig. 1). The reduction of the carbon source was 87% for SRB-L, 82% for SRB-F and 78% for SRB-G. The higher sulfate reduction associated with higher carbon utilization observed with the enrichment SRB-L might be due to relatively higher metabolic ability of SRB towards lactate than glucose and fructose. Few studies also mentioned that the type of carbon source has an impact on

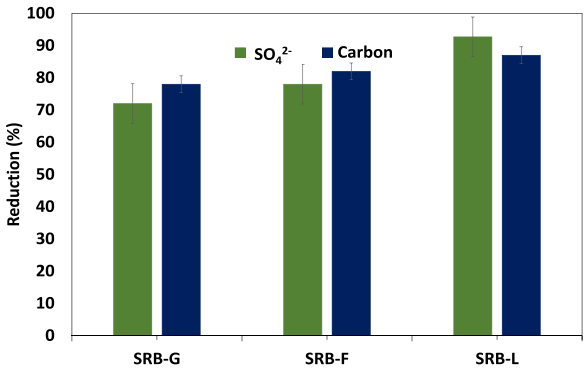

Fig. 1. Sulfate reduction and carbon utilization profiles of sulfate reducing bacteria (SRB) in enrichment cultures fed with different carbon substrates designated as SRB-L (L-lactate fed), SRB-G (G-glucose fed) and SRB-F (F-fructose fed) represented in percentage.

the rate of sulfate reduction and sulfide formation [25]. This is because, both fermentative bacteria (FB) and oxidative SRB can compete for lactate as carbon substrate in the presence of sulfate. However, the sulfate concentration determines the utilization of carbon between FB and SRB. When the sulfate concentration is higher, SRB becomes dominant over FB and vice versa [25]. In general, when lactate is used as a carbon source/electron donor by SRB, sulfate reduction is favored. As the sulfate concentration plays a major role, it is to be noted that optimization towards oxidative pathway by gradual increase in sulfate concentrations aids in limiting the occurrence of fermentative pathway, thus augmenting the biological sulfate reduction. Similarly, changes/-shifts in pH were also monitored in all the enrichments with respect to sulfate reduction. Sulfate reduction results in sulfide formation which increases pH. A rise in pH from the initial pH was observed in all enrichments, corresponding to a pH of 6.9±0.2 for SRB-L, 6.4±0.3 for SRB-F, and 6.2±0.2 for SRB-G respectively. Although different carbon substrates such as glucose, fructose and lactate were tested, lactate attained relatively higher sulfate reduction and increase in pH, and vice versa for glucose and fructose. This is in accordance with sulfate reduction reactions, which causes an increase in pH, as the sulfate reduction is a proton consuming reaction. Successfully enriched SRB-L culture was used as biocatalyst in BES reactors for rapid and increased sulfate reduction.

3.2. BES operation with enriched SRB: sulfate reduction and sulfide formation

The enriched SRB-L that performed best in terms of sulfate reduction, was chosen to be used as biocatalyst for BES operation. Two BES reactors designated as BES-1 and BES-2 were inoculated with enriched SRB-L and were fed with lactate (3.1 ± 0.1 g/l or 34.41±1.1 mM) and sulfate at a concentration of 3.2 ± 0.05 g/L or 33.9±0.6 mM. Fig. 2a and b shows the molar concentration profiles of sulfate and sulfide along the operation at various batches of BES-1 and BES-2. Reduction of sulfate to sulfide is evident with the decreasing concentration of sulfate and increasing concentration of sulfide, maintaining the sulfur mass balance in the system. Fig. 2c depicts the percentages of sulfate reduced in BES-1 and BES-2 based on the initial and final level of sulfate in a batch. Initial batch operation depicted a rapid decrease in sulfate concentration in both BES reactors of up to 98% (BES-1) and 95% (BES-2) (Fig. 2). Correspondingly, a total sulfide concentration of 37.43 mM (1.25 g/l) was formed in BES-1 (Fig. 2a), while it was 34 mM (1.1 g/l) in BES-2 (Fig. 2b). The sulfide concentration is a measure of total sulfide concentration, which includes dissolved hydrogen sulfide (H_2S) and

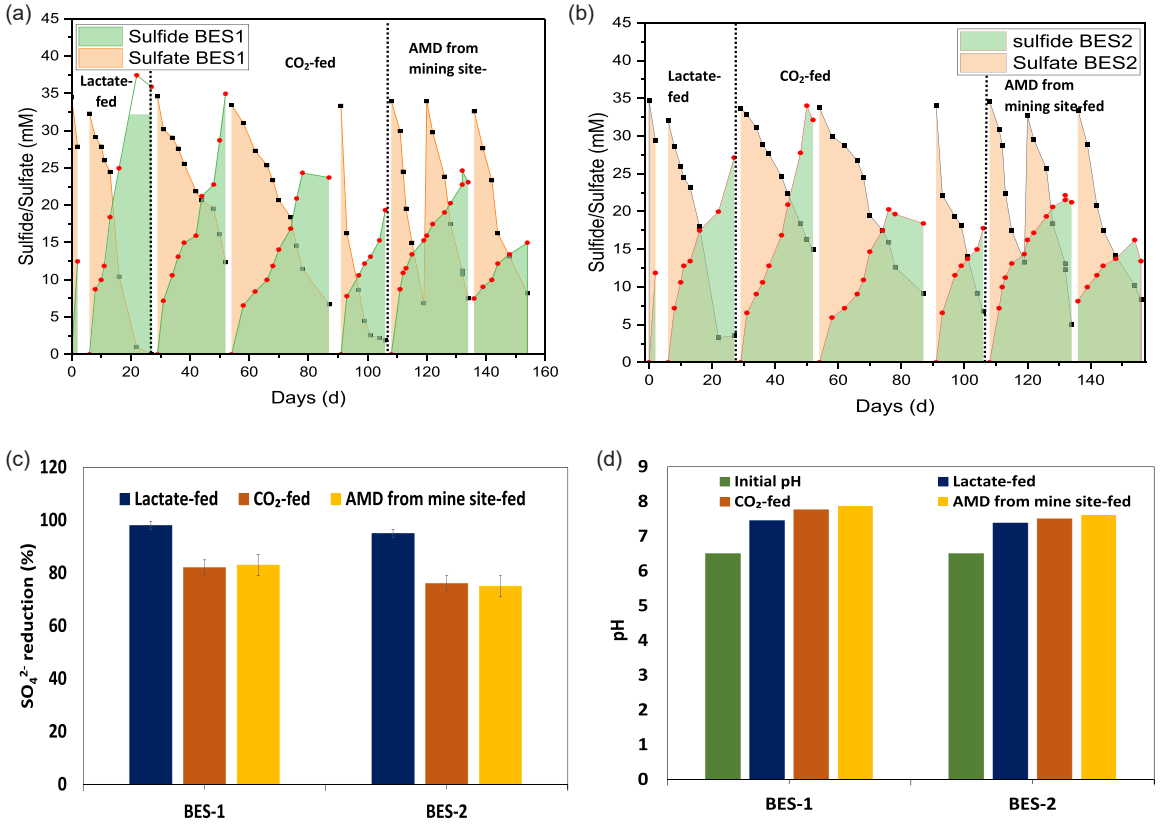

Fig. 2. Molar concentration profiles of sulfate and sulfide production in (a) BES-1 (Top left) and (b) BES-2 (Top right) at various phases of operation. Average (c) sulfate reduction efficiencies (Bottom left) and (d) pH variation (Bottom right) in BES-1 and BES-2 during lactate-fed, CO_2-fed and AMD from mining site-fed phases of operation.

bisulfide (HS^-) in the neutral to slightly alkaline pH range. Despite these two forms, sulfate reduction could yield several possible forms, such as thiosulfate, sulfite, elemental sulfur, etc. However, this study analyzed total dissolved sulfide concentration only as a result of sulfate reduction and did not analyze other forms. Concomitantly, lactate utilization was found to be 83% in BES-1, and 76% in BES-2, which is in accordance with the sulfate reduction in both BES reactors. This depicts the impact of carbon source as an electron donor in accelerating sulfate reduction reactions. Lactate also favors the growth of several other bacteria that co-exist in the inoculum besides the enriched SRB. For this reason, there is a possibility of several non-SRB lactate fermenting bacteria along SRB despite sulfate presence which has been reported in anaerobic fermenters as well as in natural environments [26–28]. Higher lactate concentration encourages non-SRB growth along with SRB thereby resulting in substantial sulfate reduction with the electrons acquired from lactate oxidation [29].

After carrying out few batch operations in BES with lactate as carbon source, SRB was allowed to function under chemolithotrophic conditions (CO_2 fed along with SAMD) by eliminating the carbon source. This approach was adopted to avoid the need of external organic substrate for an on-site AMD treatment with BES, thereby enabling a more straightforward and practical implementation. Preliminary batch operations, after eliminating lactate, resulted in lower levels of sulfate reduction which is attributed to a number of reasons, such as the need of bacterial adaptation to chemolithoautotrophic conditions, the lack of immediately available electron donor, metabolic pathway shifts, changes in bacterial community due to switch towards chemolithotrophic conditions, etc. Fig. 2c depicts the maximum sulfate reduction of 65.3±2% in BES-1 and 58.3±3% in BES-2 during the first two batches with only CO_2 as carbon source. These values are relatively lower as compared to the percentages of sulfate reduction with lactate supplementation. Correspondingly, sulfide formation of 21.8±2.5 mM (0.6 g/l) in BES-1 and 19.03±1.2 mM (0.43 g/l) in BES-2 were observed on average of two batches with CO_2 (Fig. 2a & 2b). Other intermediate forms of sulfate reduced species might have been formed, but they were not measured during the study. During the next cycles with CO_2 and 50% AMD from mine site, a steady progress was observed in terms of sulfate reduction and sulfide production in both BESs (BES-1: SO_4^{2-}: 82%, sulfide: 24.32 mM i.e. 0.79 g/l; BES-2: SO_4^{2-}: 76%, sulfide: 20.27 mM i.e. 0.65 g/l), which indicates acclimatization of bacteria to organic carbon limited conditions as well as development/enrichment of microbiome community that can sustain under chemolithoautotrophic conditions (Fig. 2 and Fig. S1). Limiting the organic carbon and supplementing the electron source via set cathode potential also favored the growth of chemolithoautotrophs (such as SRB, sulfur oxidizing bacteria, homoacetogens, Archaea, iron oxidizing bacteria, etc.) while restricting the growth of FB (see Section 3.6 for details). Few studies mentioned that some microbes such as *Desulfosporosinus orientis* [DSM-765], and *Desulfovibrio piger* [DSM-749] can utilize CO_2 and H_2 to reduce sulfate [30]. It is also interesting to note that the sulfate reduction was maintained significantly under chemolithoautotrophic conditions (Fig. 2 and Fig. S1). A prime reason for the sulfate reduction in chemolithoautotrophy could be attributed to the phenomenon called 'electro-autotrophic sulfate reduction'. During this phenomenon, SRB acquires electrons directly from electrode which is used for sulfate reduction to sulfide through an intermediary sulfite formation. The whole reaction requires eight electrons per sulfate reduction wherein, two out of eight are required at intermediary sulfite formation. Other possible ways of sulfate reduction in BES might be via mediated electron transfer through the hydrogen produced either biologically or abiotically during the process. A maximum of 3.1–3.2 g/l initial sulfate concentration was supplemented to both BES reactors, wherein the sulfate reduction is observed, however at a gradual pace with simultaneous acetogenesis. This indicates that the electron distribution might have occurred between SRB and acetogens thus performing both the processes of sulfate reduction and acetate synthesis. Details about microbial synergy for sulfate reduction and acetic acid synthesis is discussed in detail in section 3.7. Although the level of sulfate reduction seems promising, the retention times for the operation were a bit extended. The dominance of acetogenesis over sulfidogenesis processes can be based on several parameters such as change in pH, applied voltage, hydrogen formation, electron transfer routes etc.

During BES operation, changes/shifts in pH were noticed in both BES reactors with respect to sulfate reduction. Initial pH for both BES was set at pH 6.5 and were allowed to operate at applied voltages of −1.0 V (BES-1) and −0.8 V (BES-2). With respect to sulfate reduction, increase in pH was observed reaching up to 7.83±0.4 in BES-1 and 7.66±0.3 in BES-2 during all the phases starting initially from pH 6.5, indicating proton consumption towards sulfidogenesis (Fig. 2d). SRB grow in a wide range of pH of 5– 8 that facilitate biological sulfate reduction as observed in several studies [6]. Few reports indicate that low pH is best for sulfate reduction and sulfide formation, but lower cell growth is observed as much energy is used only for proton pumping and maintaining the pH homeostasis [31]. Nevertheless, operation at near neutral and neutral pH has an advantage of cell growth and sulfate reduction, limiting the energy dissipation for other processes. Also, optimization of pH leads to better growth and sulfate removal. This study noticed a gradual increase in pH with respect to sulfide generation and simultaneous acetate synthesis. It is to be noted that gaseous H_2S formation tends to occur at a pH range below 7, which is not observed in this study (pH >7) and remained as HS- (hydrosulfide) above 7 which entangles with metals such as 'Fe' to form several forms of iron sulfide (FeS^0, Fe_2S, FeS_2 etc.) based on the prevailing pH. Details about the iron sulfide forms and structure will be discussed in the sections below detailing the structural and elemental characterization results. Several reports mention that pH above or near 8 can facilitate the precipitation of iron sulfides [32]. Eventually pH was regulated due to the buffering within the system which comprises CO_2, bicarbonate, phosphates or other ionic forms that can help in buffering action.

3.3. Organic metabolites synthesis

In BES organic metabolites formation was observed alongside sulfate reduction. Several carboxylic acids were observed ranging from C2 (acetic acid) to C4 (butyric acid), of which C2 was the most predominant. Initial operation of BES with lactate as carbon source facilitated sulfate reduction along with formation of propionate and acetate as intermediary compounds. It is known that lactate metabolism yields acetate and CO_2 via oxidative pathway, while it can also form propionate via fermentative pathway [25]. The choice of metabolic pathway to form propionate and acetate is dependent on lactate and sulfate concentrations. It was observed in several studies that lactate fermentation yields propionate and acetate simultaneously during sulfate limiting conditions, where the FB becomes dominant [25–30]. On the contrary, this study noticed both acetate and propionate synthesis during lactate metabolism with simultaneous sulfate reduction, which implies that partly electrons from lactate metabolism were utilized for sulfate reduction, while the other electron requirement might have been sufficed by H_2 generated during BES operation under poised cathodic potentials. During the initial cycles (lactate fed conditions), organic metabolites production was low and a gradual increase in propionate and acetate synthesis was noticed in the subsequent cycles of both BES fed with lactate and sulfate. A maximum of 0.98±0.3 g/l and 1.23 ±0.3 g/l of propionate and acetate were synthesized in BES-2, while it was 1.0±0.2 g/l and 0.6±0.2 g/l of acetate and propionate respectively in BES-1. Differences in propionate and acetate concentrations in BES-1 and BES-2 might be due to the variation in cathode potentials that might have triggered in facilitating oxidative and fermentative pathways along with differences in hydrogen production. A slight consumption of organic metabolites was observed in both the BESs (0.6±0.5 g/l) as depicted in Fig. 3.

The second phase of operation (CO_2 fed conditions) wherein the

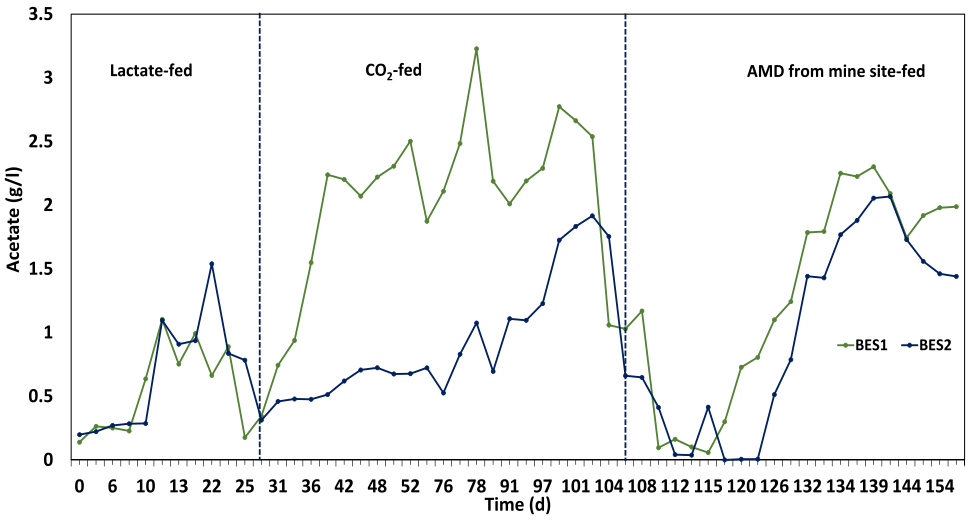

Fig. 3. Acetate electrosynthesis in BES-1 and BES-2 at various phases demarcated as lactate-fed, CO_2-fed, and AMD from mining site-fed conditions operated for sulfate reduction.

carbon source was depleted (no lactate supplementation), CO_2 was provided with recirculation via gas bags. During this phase, acetate was the predominant organic metabolite synthesized simultaneously with sulfate reduction. Initially, acetate concentration was in the range of 0.7 ±0.3 g/l which gradually increased to 2.5±0.4 g/l in BES-1, while in BES-2, acetate concentration was 0.3±0.8 g/l and gradually increased to 0.9±0.3 g/l. After acclimatization, significant improvement in the acetate yields up to 3.0±0.2 g/l and 1.1±0.12 g/l were observed in BES-1 and BES-2 respectively. High concentration of acetate correlates to the presence of chemolithoautotrophic bacteria coexisting with SRB. In general, SRB can synthesize acetate with sulfate reduction in the presence of an organic electron donor, preferably lactate as observed in several studies of sulfidogenesis [33]. Since the phase without organic carbon supplementation had acetate in the major fraction, this might have been a result due to the metabolic action of chemolithoautotrophic homoacetogenic bacteria that can grow well under the favorable conditions of CO_2 and H_2 availability. These homoacetogenic bacteria can function in synergy with SRB to produce acetate, while SRB can utilize the electrons or acetate for sulfate reduction. Besides homoacetogenic bacteria, methanogenic microbiome communities can also function in synergy with SRB where electron distribution takes place towards specific metabolic pathways [33–38]. Subsequent BES operation with SAMD and AMD from mine site mixed at 50:50 depicted reduction in acetic acid concentration, which might have been consumed by any of the possible bacteria groups such as heterotrophic SRB, acetoclastic methanogens, fermentative bacteria, heterotrophic homoacetogens etc. From the sulfate reduction profiles, it is perceived that acetate was utilized for sulfate reduction, as the continuous reduction in sulfate concentration was observed during days 101–120. Interestingly, microbiome community analysis (discussed in Section 3.6 in detail) also identified dominant groups of bacteria which are SRB, homoacetogens, methanogens and syntrophic bacteria that could have functioned in synergy among them in electron utilization towards multiple functions of sulfidogenesis, acetogenesis and methanogenesis (not measured in the study). There is a probability of SRB switching its metabolism into heterotrophic mode that can utilize acetate and perform sulfate reduction. During the days from 111 to 115, acetate concentration was declining and was almost negligible in BES-1, while the same was observed from day 111–122 in BES-2, which indicates acetate consumption amongst the microbial groups. Later, there was a steady increase in the concentration of acetate from day 116 in BES-1 and from day 123 in BES-2, which reached 2.12±0.23 g/l on day 139 in BES-1 and 1.9±0.2 g/l on day 142 in BES-2. After this, acetate concentration again showed a slight decrease in both the BESs which continued in BES-2, while BES-1 depicted an increase in acetate after 144th day. The periods or phases of decrease in acetate concentration might be ascribed to its consumption by other bacteria groups, while the increase is related to synthesis by homoacetogenic bacteria. Details about bacteria groups and their probable synergistic relationship are discussed under microbial community analysis in below sections.

3.4. Current demand

Bioelectrochemical operation for sulfate reduction noticed responsive currents generation to applied voltages in BES-1 and BES-2 (Fig. S2). Higher reduction currents were observed in BES-1 in comparison to BES-2 throughout the operation irrespective of carbon source being organic/inorganic. This difference in currents might have been due to the difference in applied voltages. Initially, the reduction currents were −10 mA and −5 mA in BES-1 and BES-2 respectively, which gradually increased with respect to time. This indicates the adaptability of bacteria to sulfate rich wastewater, signifying the catalytic activity of bacteria participating in electron transfer for enhanced current generation. Initial phase of operation with organic carbon (lactate fed conditions) depicted reduction currents in the range of −5 to −17 mA in BES-1, while it was −3 to −10 mA in BES-2. This initial phase displayed almost similar range of reduction currents in BES-1 with marginally higher reduction currents than BES-2. This could have been due to variation in applied cathode potentials in both BESs.

In the second phase of BES operation with chemolithoautotrophic conditions (CO_2 fed conditions), reduction currents were initially low, which gradually increased indicating the adaptability of bacteria to inorganic carbon and facilitating sulfate reduction efficiently. Thereafter, a gradual and steady increase in reduction catalytic currents was noticed in both BESs (BES-1: −70 mA; BES-2: −58 mA), while SAMD and AMD from mine site (50:50) operation also displayed high reduction currents of −75±6 mA in BES-1 and -68±5 mA in BES-2 respectively which were quite high compared to regular MES/BES operation. Higher reduction currents can be attributed to metal deposition or metal sulfide, mainly FeS deposition on cathode surface as sulfate reduction forms sulfide, and the possible form is FeS from $FeSO_4$ (discussed under SEM analysis in Section 3.5). Several studies reported the importance of iron sulfide particles having conductive properties that promote extracellular electron transfer [19]. As the batch cycles were conducted repeatedly, FeS deposition on electrode surface might have continued, which resulted in high reduction currents in chrono amperometry analysis.

This is also in support with the results obtained with scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) analysis which is discussed in the sections below. We speculate that the increase in deposition on electrode surface is in direct correlation with the increased reduction currents implying the potentiality of FeS particles in augmented electron transfer. Although multiple metabolic interactions are happening which can cause an increase in electron bifurcation towards many processes, sulfate reduction was still progressing along with acetic acid production. It is agreeable that synergy might have taken place between/among bacteria groups for electron transfer, but it is very important to understand the significance of FeS particles deposition which might have acted as electronic conduits in promoting electron transfer extracellularly. A recent study reported that physicochemical properties of FeS particles synthesized biologically play crucial role in biobased energy production under physical or thermodynamically unfavorable conditions which encourages them to undergo magnetotaxis or by promoting electron transport to solid electron acceptors [19]. These FeS particles might also act as electronic conduits which can promote direct interspecies electron transfer (DIET) between different bacteria, as the conductive particles were reported to have these features [34]. The conductive particles also hinder electron losses that majorly occur in BES/MES and encourage development of higher current densities, leading to continuous electron availability that facilitates electron shuttling in multiple metabolic processes among diverse bacteria groups [35]. Although it is unclear whether electron transfer is either hydrogen mediated or directly from electrode, FeS particles can act as an electron shuttle thereby encouraging sulfidogenesis and acetogenesis. Electrotrophy might have been a key phenomenon occurred during BES via FeS particles deposited on electrode surface as evident through reduction current profiles accompanied with simultaneous sulfate reduction and acetate synthesis without any organic electron donor. It is also possible that FeS particles can be synthesized intra and extracellularly, and these could deposit on the cell surface which can perform rapid electron transfer towards and from electrode. Other possible metal sulfide particles in AMD from mine site could be zinc sulfide (ZnS) particles, as zinc was dominant metal among other metals available. Since the operation was carried in dilution with SAMD (50:50), other metal sulfide particles than FeS from SAMD is given limited attention in this study.

3.5. Morphological, structural, and elemental characterization

The precipitates formed in BES after sulfate reduction were analyzed for the composition of metals/elements using EDS for the inorganic carbon phase fed with SAMD (Fig. 4). Both the BES displayed the presence of elements such as iron, sulfur, carbon in major fraction, while other elements such as oxygen were also observed. BES-1 noticed relatively higher weight fraction of iron and sulfur (Fe: 15.9%, S: 6.2%) than BES-2 (Fe: 6.1%, S: 1.0%) which is also in synchrony with the sulfate reduction that formed sulfides. EDS spectra displayed Fe and S in relatively higher fractions and speculate the formation of mackinawite and other forms of FeS. Besides these, oxygen and carbon were also appeared to be in major fraction in the EDS spectrum. These might have been the result of bacterial biomass and cathode material or from the hydroxides/organic metabolites complex formed with the iron-sulfide particles.

Besides, surface morphology of the metal sulfide particles of both the BESs was analyzed using scanning electron microscopy (SEM) for the inorganic carbon phase fed with SAMD. The chemistry of iron-sulfide systems is intricate which projects the possibility of several different forms based on prevailing pH and oxidation reduction potentials (ORP). At neutral or near neutral pH (7.1– 7.4), mackinawite (iron nickel sulfide) was reported to be a major precipitate, and at moderate acidic range pH (5.4– 5.6), amorphous ferrous sulfide was the most probable form [32]. The other forms that are possible are cubic pyrite, Troilite or pyrrhotite (FeS), greigite (Fe_3S_4) and other forms of pyrite (marcasite FeS_2). BES-1 depicted a typical pattern of crystal morphology which resembles the mackinawite form of FeS. Besides these, cubic form of FeS (pyrite) is also observed in BES-1. Nevertheless, as the pH after sulfidogenesis appeared to be above 7, we speculate that mackinawite might have been the possible iron sulfide form prevailed in BES. An interesting observation was the structural alterations on cell surface of bacteria which occurred in response to iron-sulfide complex, triggering stress induced changes [32]. One study reported that cell surface compounds of microbes or extracellular material significantly favors aggregation of particles and growth of mackinawite [32]. Biogenic iron sulfide crystallizes mostly as mackinawite in comparison to abiotic systems [32]. The ultimate properties and morphology of iron sulfide particles is dependent on the binding of Fe^{2+} to microbial compounds prior to the precipitation of FeS. Similarly, BES-2 also depicted iron sulfide particles formation with spherical and disc shaped structures which are presumed to be mackinawite and cubic FeS particles. Mackinawite form of FeS displays potential conductive properties that facilitates accelerated electron transfer, which is in support with the results obtained in this study in terms of electron utilization towards acetogenesis, sulfidogenesis and other metabolic requirements of multiple bacteria groups.

3.6. Microbial community structure

Microbiome community dynamics of cathode biofilm and suspension were assessed in both the BES reactors which showed the abundance of different bacteria groups (Fig. 5). Microbiome community depicted dynamics with respect to time which have occurred due to prevailing operational conditions. Parent inoculum (D3) represented bacteria comprising of *Methanobacterium, Planococcus, Hydrogenispora (Class: Limnochordia), Clostridium* and *Romboutsia* in abundance while it also consisted of *Terrisporobacter*, *Bacillus*, *Paraclostridium*, *Sporomusa*, *Petrimonas sulfuriphila, Lysinibacillus* and *other Clostridium* in lower abundance. It is interesting to see the dynamics in microbiome community in both BESs, which represented specific bacteria evolved/enriched over a period which are remarkably distinct from the parent inoculum. An overview of bacteria diversity revealed the phylum Firmicutes being the major fraction and a low occurrence of the phylum Proteobacteria which could be attributed to several reasons such as prevalence of chemolithoautotrophic conditions that encourage the growth of the former phylum and favor acetogenesis, sulfate reduction, hydrogenesis, hydrogen utilization, electro autotrophy etc. over the latter phylum. BES-1 and BES-2 represented few microbial groups in common, while other microbial groups are distinct to each other.

D4 represents the microbial community of suspension during lactate and CO_2 fed conditions of BES-1, and is majorly comprised of *Methanobacterium* followed by *Clostridium, Petrimonas, Cellulosimicrobium* and *Bacillus*, while other bacteria groups appeared in low fraction. Among these, *Methanobacterium* fraction appeared to increase compared to the parental inoculum, signifying its enrichment. *Methanobacterium* is reported to have several functions such as production of methane and hydrogen, interspecies electron transfer, and synergistic interaction with other microbes etc. which would help for sulfate reduction. Pozo et al. reported that *Methanobacterium* enriched in their study for sulfate reduction in AMD significantly contributed to hydrogen production that in turn aided reduction of sulfate by acting as an electron donor under chemolithoautotrophic conditions [36]. In our study acetogenesis was observed, which could have occurred via syntrophic interaction between/among several bacteria groups such as homoacetogens, *Methanobacterium* and sulfate reducers. *Methanobacterium*, might have played a role in interspecies electron transfer favoring sulfate reduction and acetogenesis. Although it is known that external poised voltage acts as an electron donor steering the bioelectrochemical reactions for hydrogen production from the electrode, presence of *Methanobacterium* further augments the reduction reactions by facilitating hydrogen production. Other bacteria, namely *Cellulosimicrobium* is observed, which is reported for metal reduction largely, nevertheless, this bacterium possess sulfur metabolism capability [37]. *Petrimonas sulfuriphila* is majorly involved in sulfur metabolism but is reported to coexist with

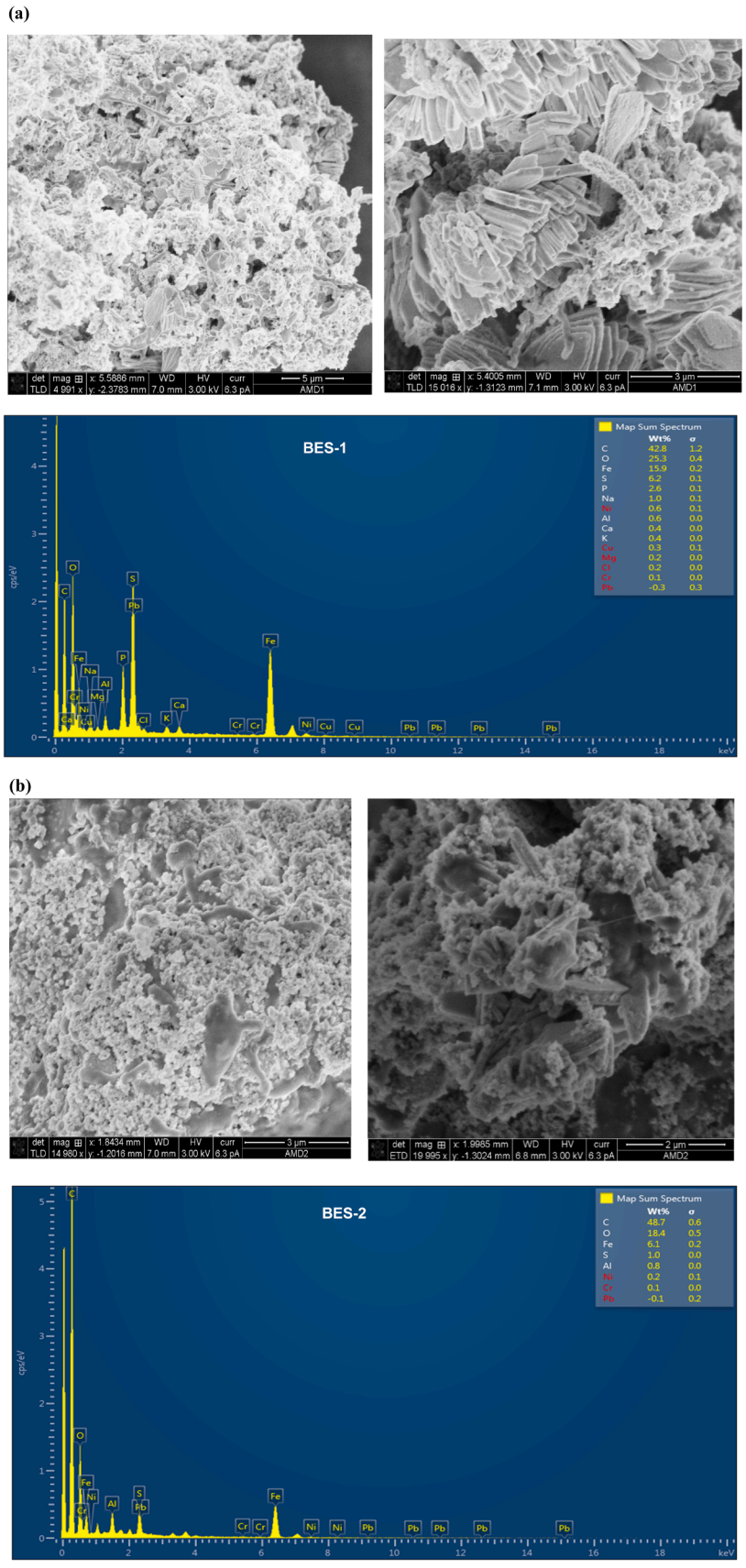

Fig. 4. Scanning electron microscopy (SEM) and Energy dispersive spectroscopy (EDS) analyses of precipitates obtained in (a) BES-1 and (b) BES-2 during CO_2 fed phase with SAMD comprising of $FeSO_4$ as source of sulfate.

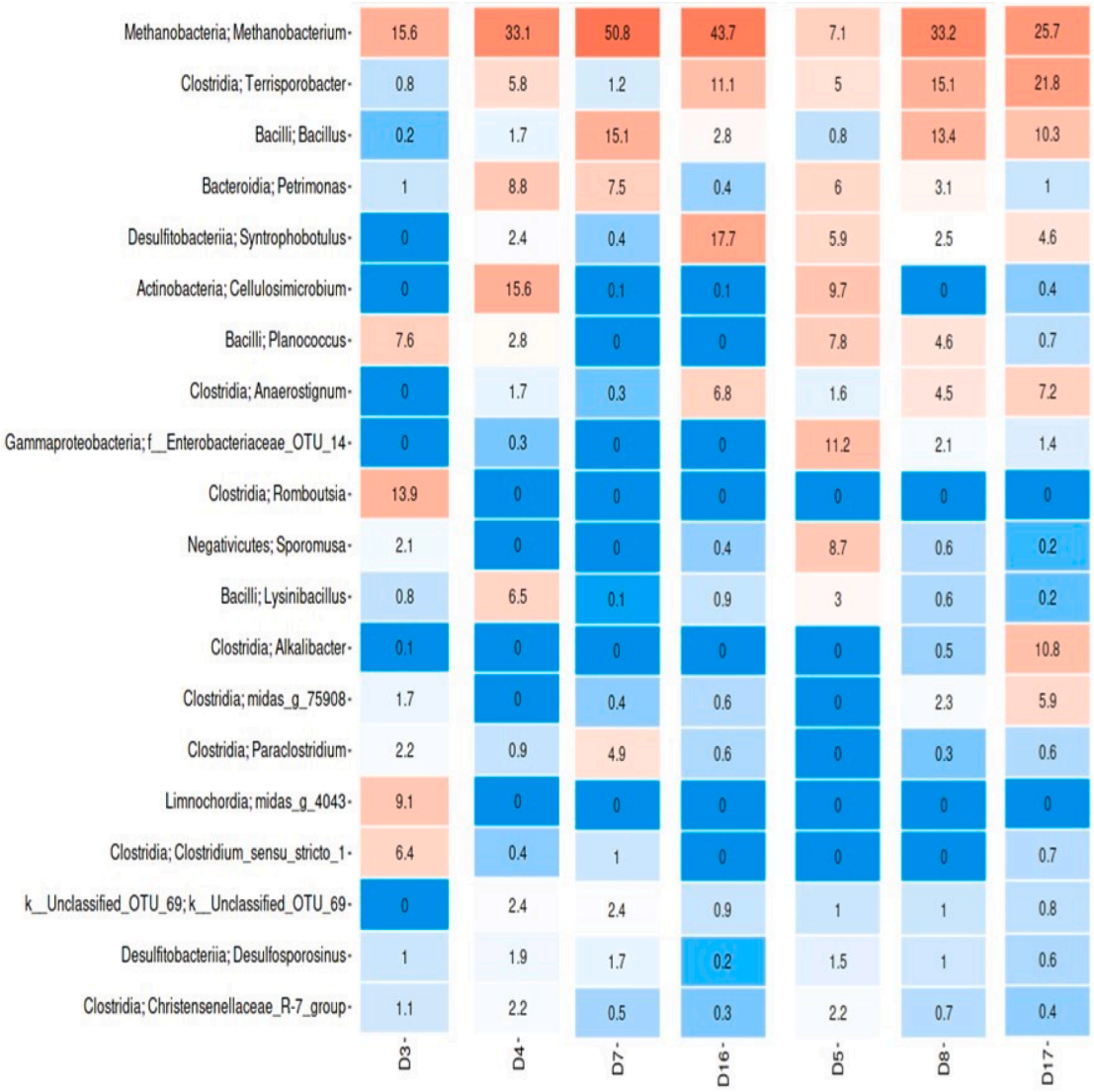

Fig. 5. Microbial community analysis of bacteria in BES-1 and BES-2 at various phases of operation with reference to parent inoculum represented with the D-labeled codes (D3: Parent inoculum; D4: Bacteria from lactate-fed and CO_2-fed conditions of BES-1; D7: Bacteria from AMD from mining site-fed conditions of BES-1; D16: Biofilm from cathode electrode of BES-1 during AMD from mining site-fed conditions; D5: Bacteria from lactate-fed and CO_2-fed conditions of BES-2; D8: Bacteria from AMD from mining site-fed conditions of BES-2; D17: Biofilm from cathode electrode of BES-2 during AMD from mining site-fed conditions).

sulfate reducers [38]. This is also reported to function in synergy with other bacteria for facilitating sulfate reduction [38]. Other than these bacteria, *Clostridium* is the most dominantly observed genus that would have favored acetogenesis as well as sulfate reduction by up taking cathodic hydrogen [42]. Interesting to note is the presence of bacteria, such as *Syntrophobotulus*, which was not identified in parent inoculum, but got enriched over time due to prevailing conditions, while *Desulfosporosinus* increased in number which also indicates their adaptability under existing conditions of high sulfate concentration. D7 which represents BES-1 microbial community during AMD from mine site fed conditions after prolonged operation indicated enrichment of same bacteria that was observed in D4 (mid adaptation phase), while it also shows emergence of new bacteria groups. *Methanobacterium* fraction is enriched greatly later in BES-1 (see D7) to 50.8% which was 33.1% at the start of operation (See in D4). Methane production was not observed during the experiment but high abundance of *Methanobacterium* signifies its role in favoring autotrophic biohydrogen production. Similar result was also reported in Pozo et al., 2015 where the highest abundance of *Methanobacterium* was observed and catalyzed the hydrogen production instead of methane production [39]. Besides, *Bacillus* genus enriched in higher fraction when compared to D4, which can be attributed to the metabolic and survival flexibility of *Bacillus* as a facultative anaerobe that could perform several functions including sulfate reduction under the simulated conditions. *Paraclostridium* is another genus which is significantly enriched in BES-1 and might be ascribed to its sulfate reduction capability and ability to thrive in sulfate rich environments. Several studies reported the function of *Paraclostridium* capable of reducing sulfate and other compounds in wastewater environment [40, 41]. On the contrary, bacteria belonging to genus *Clostridium* enriched in later stage (D7) were less abundant at D4, while others such as those belonging to genus Planococcus, Lysinibacillus decreased in D7. Microbiome community analyzed on cathode electrode (D16) during AMD from mine site fed conditions appeared interesting and indicated the dominant presence of *Methanobacterium* > *Syntrophobotulus* > *Clostridia* (*Anaerostignum* and *Terrisporobacter*), while it also had genus *Bacillus, Sporomusa*, *Desulfosporosinus* and *Clostridia* in low fraction. Although the dominance of *Methanobacterium* was observed on electrode, methane production was not observed as discussed previously. The bio-electrocatalytic hydrogen production could be the most possible role of *Methanobacterium* and it could establish syntrophy with sulfate

reducers and acetogens. Conventional sulfur related microbes such as *Desulfosporosinus* were partly abundant but other unconventional genera such as *Syntrophobotulus* and *Clostridium* were also most likely responsible for sulfate reduction and acetogenesis. Overall, microbiome community on cathode indicates the dominance of biofilm communities that might have functioned towards facilitating sulfate reduction via electroautotrophic phenomena, which is unique to the poised electrode conditions in BES. Each of the microbial genus identified on electrode have specific metabolic capabilities. Sulfidogenesis and acetogenesis observed in this study might be ascribed to the function of synergy among several microbial genera which in turn aided in improving the process efficiency and resilient functioning of the established microbiome. The synergistic behavior assumed to have occurred is discussed below in detail.

D5, D8 and D17 indicates microbiome diversity of BES-2. D5 is the suspension microbiome during lactate and CO_2 fed conditions of BES-2, and depicted abundance of phylum γ-Proteobacteria which is specific to BES-2, while it is absent in BES-1 at all the phases. Besides, it also represented abundance of bacteria genus *Planococcus, Sporomusa, Petrimonas, Terrisporobacter, Desulfosporosinus, Lysinibacillus* and *Methanobacterium* which were enriched in higher fraction when compared to parental inoculum. On the other hand, the newly evolved microbiome community in BES-2 which was not present in parental inoculum contained genera *Syntrophobotulus, Cellulosimicrobium* and *Anaerostignum* belonging to class Clostridia. These were also enriched in BES-1 and are believed to have functioned towards sulfidogenesis and acetogenesis in BES-2 as well. It is interesting to note that genus *Liminochondia, Alkalibacter* and bacteria of class Clostridia that were present in parental inoculum were absent in BES-1 and BES-2, indicating the impact of subjected environments in enriching one and suppressing the other microbial communities. D8 represent microbial community of BES-2 during AMD from mine site fed conditions after prolonged operation which revealed *Methanobacterium* enriched in significantly high fraction, and also had bacteria of genus *Terrisporobacter, Bacillus*, *Planococcus* and *Anaerostignum*. D17 represents microbiome community of cathode electrode (biofilm) of BES-2 during AMD from mine site fed conditions, which indicated dominance of *Methanobacterium* as well as genus *Terrisporobacter, Bacillus, Anaerostignum, Syntrophobotulus, Alkalibacter* and *Clostridium*, while it also had the presence of genus *Desulfosporosinus, Sporomusa* and class *γ-Proteobacteria,* which might have functioned independently or in synergy contributing towards overall enhancement in process efficiency. Unconventional genera such as *syntrophobutulus*, *Bacillus* and *Clostridium* were most likely responsible for syntrophic sulfate reduction and acetogenesis. Syntrophy among electrotrophs, HER catalysis, sulfidogenesis and acetogenesis established in the microbiome is contributing towards overall enhancement of sulfate reduction process. This microbial community structure provides indication of bio-electrocatalytic syntrophic association among the bacterial functional group to carry out sulfate reduction and CO_2 reduction. The enrichment of class γ-Proteobacteria which will have majority of bacteria with electrogenic capabilities as well as diverse metabolic functions that might have aided in sulfate reduction in synergy with other bacteria in close association.

3.6.1. Microbial synergy

Microbial community observed in this study depicted dominant presence of *Methanobacterium* on electrode and in suspension in BES-1 and 2 reactors. More commonly, it is expected that presence of *Methanobacterium* will result in methane production. However, this study did not observe/analyze methane production, instead it is in support with the previous studies that identified the enrichment of *Methanobacterium* contributing towards hydrogen production, specifically in bio-electrochemical systems operated for sulfate reduction [2]. The microbiome community appeared to be interesting in this study, as it did not reveal potential sulfate reducers in abundance, despite of attaining higher sulfate reduction. This could have happened due to the synergistic interactions between several bacteria that ultimately facilitated sulfate reduction. Majorly, three functions i.e., sulfidogenesis, acetogenesis and hydrogenogenesis were observed during BES operation. It is known that homoacetogens, methanogens and sulfate reducers function in synergy with each other depending on sulfate concentration. Electron donor in these experiments is not organic carbon but is bio-electrocatalytically generated hydrogen which is expected to aid in sulfate reduction. Besides this, presence of *Methanobacterium* in high fraction on electrode and suspension in both BES reactors is suggestive of its involvement in hydrogen production, thereby aiding for autotrophic sulfate reduction by other group of bacteria. Besides, acetate synthesis observed in BES is an indication of homoacetogenesis carried out by genera *Sporomusa* and *Clostridium* that utilizes the supplemented CO_2 [42]. It is presumed that there might have been a competition for hydrogen utilization for both sulfidogenesis and acetogenesis in which *Methanobacterium* could have significantly catalyzed hydrogen production and supported the high hydrogen demand for the overall processes. Also, other significant genus identified is *Syntrophobotulus* which is reported for functioning in synergy with other bacteria such as SRB, homoacetogens and methanogens, and is also capable of oxidizing fatty acids and releasing H_2 and CO_2 [43–45]. Nevertheless, *Methanobacterium* is also reported to have the DIET function, majorly reported for methanogenesis, however, could also facilitate other functions by aiding in interspecies electron transfer for the synergy among multiple functional groups of bacteria [46]. As this study also noticed the presence of FeS particles analyzed through SEM and EDS, it is speculated that these particles possessing conductive properties might have aided in augmented electron transfer by acting as electronic conduits for DIET or as electron shuttle. Another point to be noted is the role of sulfate concentration on the dominant microbial groups i.e. *Methanobacterium*, *Syntrophobotulus, Bacillus* and other *Clostridia* that are believed to have functioned in synergy for sulfate reduction and acetate synthesis. The comparison of sulfate concentration in determining the dominance of sulfate reducing, acetate and methane producing microbial communities has been discussed in several studies when organic carbon (mostly lactate) was provided as an electron donor [45]. Our study differs from the above, as it is an electroautotrophic sulfate reduction driven by electricity to provide hydrogen for enabling the process of sulfate reduction. In the initial phase of operation, where lactate was fed to BES, sulfate reduction was dominant and during the later phases where lactate was eliminated and operated autotrophically, acetogenesis and sulfidogenesis were noticed parallelly, signifying electron distribution/H_2 utilization via abiotic (electrode potential controlled) or biotic means (*Methanobacterium*). During BES operation, decrease in acetate concentration was also observed, and the prevalence of genus *Syntrophobotulus* indicates that acetate synthesized in this study might have been utilized to produce H_2 and CO_2 which in turn could have been utilized by SRB for sulfate reduction or by homoacetogens for acetate synthesis. A synergy might have resulted among the microbial groups favoring sulfate reduction and acetate production.

4. Conclusion

Study demonstrated the treatment of acid mine drainage (simulated and AMD from mine site with synthetic mix) containing high sulfate load using enriched SRB in two BES (BES-1 and BES-2). Sulfate reduction coupled with sulfide production was higher in BES-1 than BES-2, which might be ascribed to difference in applied voltages that steer the process efficiency. Acetogenesis was also observed during the study which is an added advantage that was resulted due to the function of homoacetogenic bacteria on CO_2 reduction. Microbial community analysis revealed dominant presence of *Methanobacterium* and the genus *Syntrophobotulus, Bacillus* and *Clostridium* along with low abundance of *Desulfosporosinus*. SEM and EDS analysis revealed presence of FeS particles, that might have aided in efficient electron transfer towards multiple functions among diverse bacteria community. Study provided insights on to

understanding the microbial synergy for facilitating autotrophic sulfate reduction, sulfidogenesis and acetogenesis, even under the conditions of low notable sulfate reducing microbial community. This study gave leads on utilizing cathodic bioelectrochemical treatment of AMD with high sulfate load devoid of organic electron donors, with microbial synergy that facilitates high sulfate reduction efficiency.

CRediT authorship contribution statement

Annie Modestra J.: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Suman Bajracharya:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Leonidas Matsakas:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Ulrika Rova:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Paul Christakopoulos:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.scenv.2024.100106.

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