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Metal precipitation in an ethanol-fed, fixed-bed sulphate-reducing bioreactor

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ABSTRACT

A batch upflow fixed-bed sulphate-reducing bioreactor has been set up and monitored for the treatment of synthetic solutions containing divalent iron (100 mg/L and 200 mg/L), zinc (100 mg/L and 200 mg/L), copper (100 mg/L and 200 mg/L), nickel (100 mg/L and 200 mg/L) and sulphate (1700 mg/L and 2130 mg/L) at initial pH 3–3.5, using ethanol as the sole electron donor. The reactor has been operated at the theoretical stoichiometric ethanol/sulphate ratio. Complete oxidation of ethanol has been achieved through complete oxidation of the intermediately, microbially produced acetate. This is mainly attributed to the presence of *Desulfobacter postgatei* species which dominated the sulphate-reducing community in the reactor. The reduction of sulphate was limited to about 85%. Quantitative precipitation of the soluble metal ions has been achieved. XRD and SEM–EDS analyses performed on samples of the produced sludge showed poorly crystalline phases of marcasite, covellite and wurtzite as well as several mixed metal sulphides.

1. Introduction

Wastewater originating from mining and metallurgical industries is often acidic and typically characterized by a significant content of sulphate and soluble metals, such as Fe, Zn, Cu, Ni, Pb and Cd. Biological treatment of such wastewater, based on sulphate-reducing bacteria (SRB) [1,2], is a viable option due to lower cost and better sludge qualities compared to conventional chemical treatment [3]. In such processes, SRB obtain energy for cell synthesis and growth by coupling the oxidation of organic substrates or molecular hydrogen (H_2), under anaerobic conditions, to the reduction of sulphate (SO_4^{2-}) to sulphide (H_2S and HS^-) [2]. Sulphide reacts with divalent metal ions which are then sequestered from wastewater as insoluble metal sulphides in the form of various mineral phases [4]. Sulphide and bicarbonate ions, which are formed during sulphate reduction and carbon source oxidation, equilibrate into a mixture of H_2S, HS^-, S^{2-}, CO_2, HCO_3^- and CO_3^{2-}, which buffers the solution pH around neutral to slightly alkaline values [5].

However, the dissolved organic carbon content of metal-containing wastewater is very low, usually <10 mg/L [6]. Therefore, addition of a suitable carbon source and electron donor for sulphate reduction is necessary to promote biogenic H_2S production. The preferred carbon sources for SRB are low molecular-weight compounds such as organic acids (e.g. lactate, pyruvate, formate and malate), fatty acids (e.g. acetate) and alcohols (e.g. ethanol, propanol, methanol and butanol) [7,8]. Nevertheless, several other materials have been examined as alternative, cost-effective electron donors for the SRB-based sulphate reduction, such as hydrogen (coupled with CO or CO_2 as carbon source) [8–10] and various organic materials [8,11], such as molasses [5,12–15] or wastes [16,17]. The carbon/energy substrate is clearly an influential variable because of its effect on growth rate and culture composition as well as a potential effect on the economics of a full-scale industrial process.

Ethanol is proposed as a carbon source/electron donor for sulphate-reducing bacteria for several reasons, including ease of availability and low cost. Moreover, White and Gadd demonstrated that ethanol was more effective in stimulating sulphide production than lactate which, however, produced the greatest biomass [18]. Ethanol has already been tested as carbon/electron source in biosulphidogenic metal-sequestering applications in laboratory-scale batch [19], completely mixed [20], upflow anaerobic sludge blanket (UASB) [21,22], expanded granular sludge blanket (EGSB) [23], fluidized-bed [24–27] or anaerobic membrane bioreactors [28] as well as in pilot-scale plants [29].

Ethanol can be incompletely oxidized to acetate (1) or completely oxidized to CO_2 (2), depending on the SRB species [2]:

$$2CH_3CH_2OH + SO_4^{2-} \rightarrow 2CH_3COO^- + H_2S + 2H_2O \quad (1)$$

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$$2CH_3COO^- + 2SO_4^{2-} \rightarrow 4HCO_3^- + 2HS^- \quad (2)$$

Acetate oxidation (reaction (2)), which depends on the presence of acetate-utilizing SRB in the microbial consortium, has been proven the critical step, as it controls the generation of alkalinity and the residual organic content of the effluent. Acetate may also inhibit sulphate reduction at high concentration and low pH [30], being highly toxic to SRB in undissociated forms [31]. Thus, in order to avoid acetate accumulation, efforts have been made towards engineering biosulphidogenic systems by enriching cultures with acetate-utilizing bacteria [26] or by developing syntrophic sulphidogenic microbial consortia [31] or by even supplying alternative electron acceptors, such as oxygen or nitrate [32].

Apart from the minimization of the supplied materials (i.e. organic substrate) and zero discharge of soluble metals, acetate oxidation is considered a key factor for the optimization of the entire process, since it determines the neutralization potential of the acidic feeding solution and the effluent organic carbon content.

The present work examines and discusses the acclimation potential of a lactate-grown mixed culture to ethanol, the utilization potential of the produced acetate as well as the efficiency of the reactor running at stoichiometric ethanol/sulphate ratio. Experimental set-up and methodology included: (a) liquid phase monitoring for the determination of the residual organic carbon, metals and sulphate in solution; (b) bacterial community characterization and (c) sludge morphological and mineralogical characterization.

2. Materials and methods

2.1. Sulphate-reducing fixed-bed reactor

The sulphate-reducing fixed-bed reactor, operating in upflow mode, was a PVC tube (length: 30 cm; I.D.: 5 cm) which was filled with porous, sintered-glass pipes (length: 1.5 cm; wall thickness: 5 mm; specific surface: 1200 m^2/L – SintoMec®, JBL Germany), resulting in a bed height of 26 cm and reactor effective volume of 250 mL. The reactor was inoculated by transferring sufficient support material with already grown bacterial biomass from a previously operated, lactate-fed bioreactor [33]. This mixed sulphate-reducing bacterial culture has already been characterized and described in detail [34].

In order to study the acclimation potential of a lactate-grown culture to ethanol, the final microbial degradation of the intermediately produced acetate as well as the efficiency of the reactor running at stoichiometric ethanol/sulphate ratio, a batch-fed set-up was selected. The reactor operated continuously for 12 months at constant room temperature (25 °C). The reactor, operating in batch-fed mode, was fed from a 2 L bottle via a peristaltic pump. The feeding solution was recirculated (300 mL/h) and replaced upon exhaustion without emptying the reactor, to avoid any oxidation effects due to air inflow.

The reactor was initially fed with a variation of Postgate's medium (DSMZ GmbH, *Desulfovibrio* medium no. 63), using lactate as major electron donor. Upon the development of biofilm on the support material, the culture was acclimated to a different nutrient solution, using ethanol as sole carbon/electron source. Lactate was gradually substituted with ethanol as a percentage of the total COD fed to the system as shown in Fig. 1. The electron donor change procedure was completed in 30 days.

Following, the reactor was fed with synthetic, sulphate-rich and metal-bearing solution containing divalent iron (100 mg/L, added as $FeSO_4 \cdot 7H_2O$), zinc (100 mg/L, added as $ZnCl_2$), copper (100 mg/L, added as $CuCl_2 \cdot 2H_2O$), nickel (100 mg/L, added as $NiSO_4 \cdot 6H_2O$) and sulphate (1800 mg/L, added as Na_2SO_4, $MgSO_4 \cdot 7H_2O$ and $NiSO_4 \cdot 6H_2O$). The pH of this solution was 3–3.5.

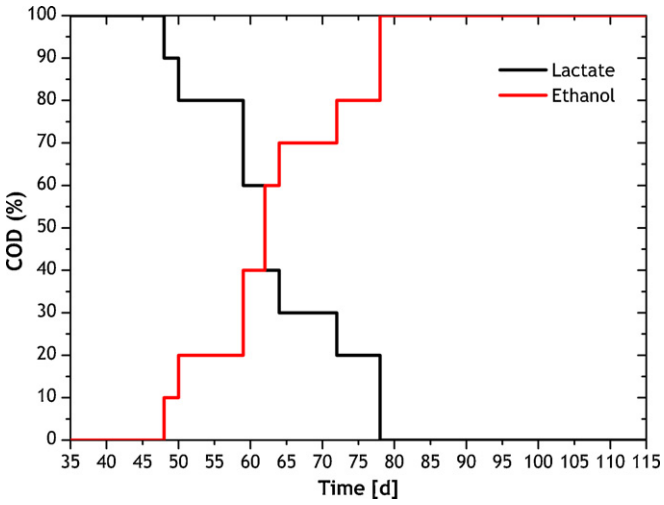

Fig. 1. Progressive shift from lactate to ethanol as electron donor.

The concentration of ethanol was reduced to the stoichiometrically required for the reduction of the sulphate in solution (reactions (1) and (2)), not taking into account ethanol assimilation for biomass growth and preservation, and all calculations thereafter were based on a molar ethanol/sulphate ratio of 0.67.

The initial concentration of the metal ions in solution was increased to 200 mg/L, leading to a corresponding increase in the initial concentration of sulphate (2130 mg/L). Therefore, the initial concentration of ethanol was increased accordingly to maintain the aforementioned stoichiometric ratio.

2.2. Liquid phase monitoring

The liquid phase parameters were monitored systematically after the reactor microbial community reached a steady behaviour each time the feeding solution content was modified. The experiments presented herein were performed at stoichiometric ethanol/sulphate ratio and at two different initial Fe–Zn–Cu–Ni concentrations: 100 mg/L (experiment 1) and 200 mg/L (experiment 2). Each experiment was repeated three times and the duration of each consecutive experimental cycle was 3 days and 4 days for experiments 1 and 2, respectively. HRT, calculated as the [effective reactor volume]/[recirculation rate] ratio for the corresponding continuous operation of the reactor, was 0.8 h.

During each experimental cycle, sampling was performed at the bottle each time the solution was renewed as well as at the reactor outlet after 5, 27, 48, 51 and 72 h (experiment 1) and 24, 27, 72, 75 and 96 h (experiment 2).

Solution pH was determined on unfiltered samples, which were then vacuum filtered through 0.45 μm sterile membranes (Whatman) before any other chemical determination. The concentrations of sulphate, TOC, copper, nickel, zinc and iron were determined as described in [33,35]. The concentrations of ethanol and acetate were determined by GC (HP 5890 II) equipped with a flame ionization detector and a 30 m × 0.32 mm (ID) capillary column coated with a 0.25 mm film of polyethylene glycol (Agilent DB-FFAP) which is recommended for the analysis of aquatic VFA solutions [24,36,37]. The temperatures of the injector and detector were 250 °C and 300 °C, respectively. The initial temperature of the oven was set at 70 °C for 3 min followed by a ramp of 20 °C/min up to the final temperature of 210 °C which was kept steady for 5 min. Nitrogen was used as a carrier gas at a flow rate of 2.2 mL/min (at 70 °C) which remained constant throughout the thermal programme of the analysis. Propanol and propionic acid were used as internal standards for ethanol and acetate, respectively.

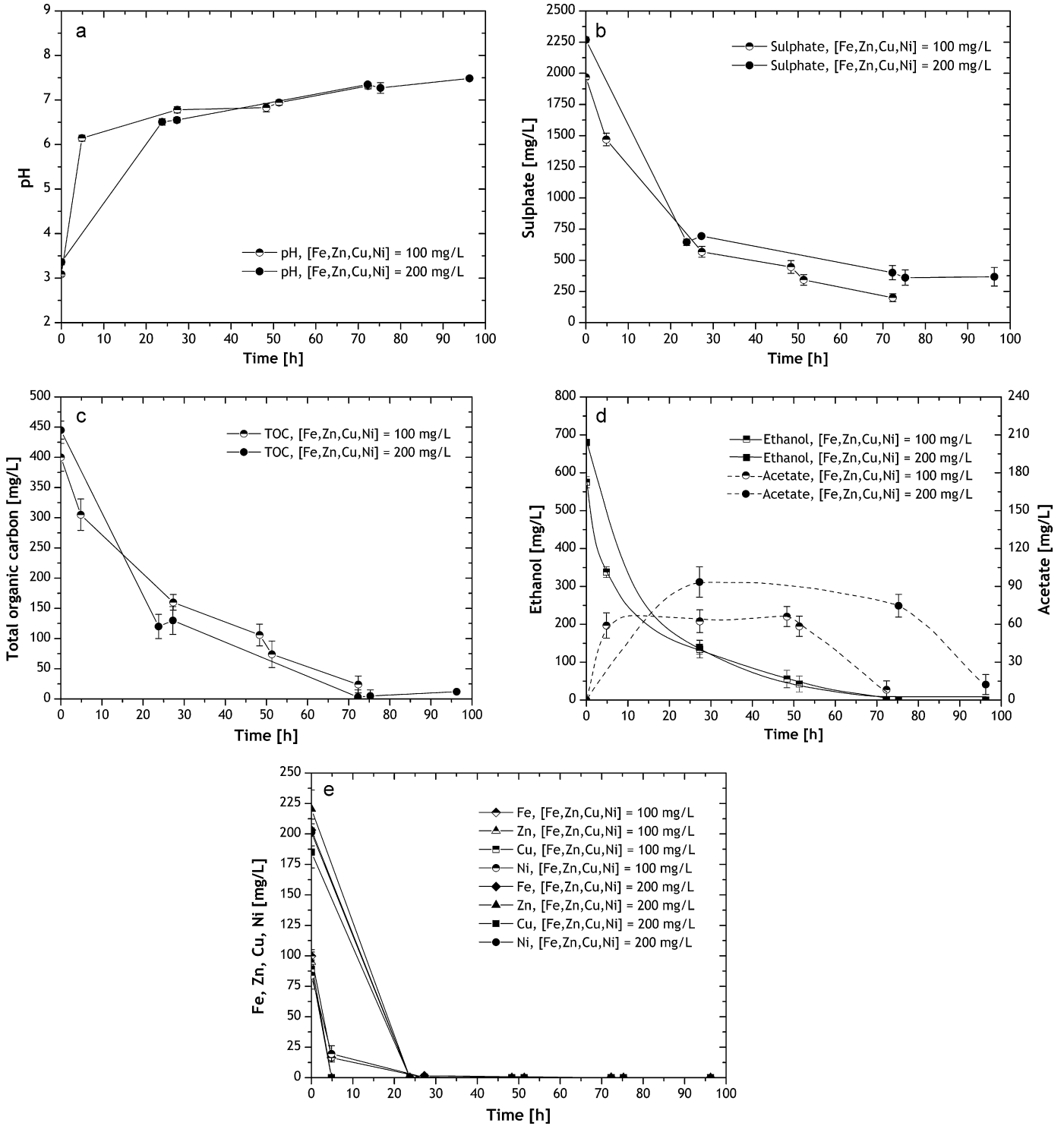

Fig. 2. (a) pH, (b) sulphate reduction, (c) total organic carbon degradation, (d) ethanol/acetate degradation and (e) metal precipitation profiles during an experimental cycle. Data points are the mean values acquired from three replicate experimental runs and triplicate analytical determinations of each sample. Error bars represent the standard deviation of the three mean values computed for each sequentially run experiment.

All analytical determinations were performed in triplicate. Based on calibration and results repeatability, the precision of the sequentially repeated determinations was estimated as: (a) pH: 1%; (b) sulphate: 5%; (c) TOC: 10%; (d) Fe, Zn, Cu, Ni: 1–5%; and (e) ethanol, acetate: 5%. The reproducibility of the results is given as standard deviation of the three mean values computed for each sequentially run experiment.

2.3. Bacterial community characterization

Effluent samples were collected in duplicate five months after commencing the reactor operation. A volume of 10 mL of each sample was vacuum filtered under sterile conditions through 0.22 μm sterile membranes (Whatman) which were kept under aseptic conditions until treatment. The diversity and the sequencing of the

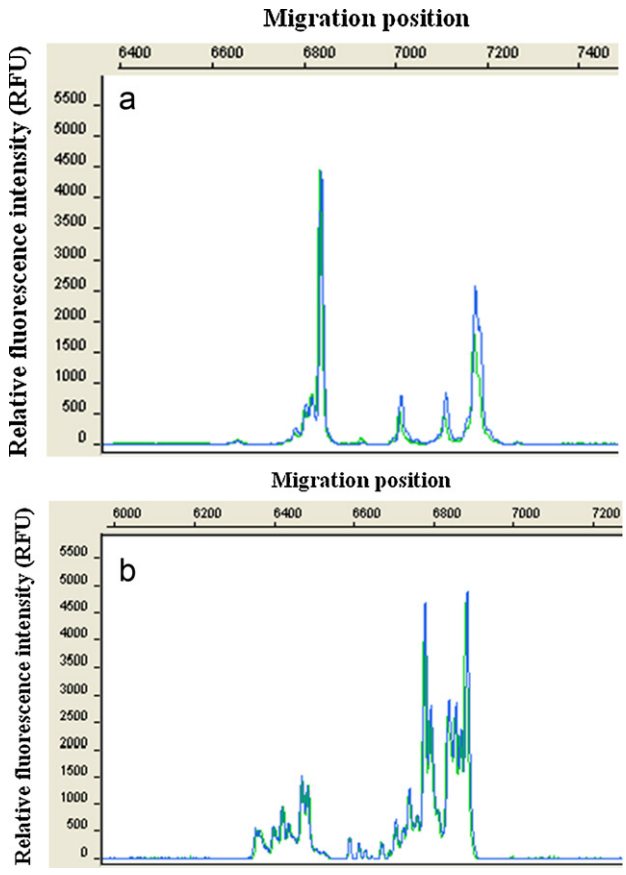

Fig. 3. Molecular CE-SSCP fingerprints of 16S rRNA genes of the bacterial community colonizing the column bed for an outlet sample taken in duplicate from (a) the ethanol-fed and (b) the original lactate-fed reactor.

bacterial community were examined as described in [34]. A *dsr*AB neighbor-joining tree was constructed from unambiguous amino acids on the calculated Kimura distances using the Jukes and Cantor method [38] implemented in the Treecon package [39]. Sequences of *dsr*AB genes are referenced in GenBank under accession numbers HQ640652–HQ640659.

2.4. Sludge characterization

Sludge was collected after the end of the reactor operation and kept refrigerated in reactor liquor until further analysis. Sludge samples were then dried in room conditions and powdered for chemical, mineralogical and morphological characterization.

Before any chemical determination, sludge samples were dried at 105 °C for 24 h. To determine volatile suspended solids in the precipitates, samples were ignited at 550 °C for 2 h and allowed to cool in a desiccator; the procedure was repeated until constant weight was obtained. To determine the metal content of the sludge, samples were acid-digested with 10 mL ultrapure HNO_3 65% (Merck) and, then, analysed by inductively coupled plasma optical emission spectroscopy (Leeman Labs, Inc.) [40]. The determinations were performed in triplicate. The obtained results for volatile suspended solids and metal content showed quite satisfactory reproducibility as the observed deviation was less than 5%.

Powder X-ray diffraction (XRD) analysis was performed on a Bruker D8 Focus X-Ray Diffractometer, operating with CuKα radiation. The samples were step-scanned from 5° to 70° (2θ), at a step of 0.02° and step time of 1 s. The major peaks of the XRD pattern were identified based on reference patterns calculated from crystal structure data (Crystallography Open Database, COD [41]).

The morphology of the precipitates was studied on a scanning electron microscope (JEOL JSM 6380-LV) in high-vacuum mode (accelerating voltage: 15 kV). Semi-quantitative elemental analysis of the SEM samples was performed by energy dispersive X-ray spectrometry (EDS system by Oxford Instruments). Powder samples were applied on adhesive tape and examined without coating.

3. Results and discussion

3.1. pH variation

Fig. 2(a) presents the pH profile during an experimental cycle. The initial pH of the fresh feeding solution was 3–3.5, whereas pH at the end of the cycle always reached a value of about 7.5 as a result of the alkalinity generated during the SRB metabolism. This indicates that the SRB metabolic process was not adversely affected nor inhibited by the initial low pH. Sulphidogenesis is, therefore, possible at low initial pH values, thus confirming the viability and the efficiency of the process when the mixed SRB community is in direct contact with acidic wastewater [31,33,42,43].

Furthermore, this pH change proves that the culture adapted successfully to the change of the electron donor as well as to the presence of four heavy metals at initial concentrations of 100 and 200 mg/L.

3.2. Organic carbon utilization and sulphate reduction

After the initial adaptation phase, the reactor operated at stoichiometric molar ethanol/sulphate ratio. The experimental results concerning the degradation of the added organic material are presented in Fig. 2(c) and (d).

Fig. 2(c) presents the degradation of the organic carbon in the feeding solution during an experimental cycle for the two different initial concentrations of the metal ions in the fresh solution. The initially fed TOC was oxidized by approximately 95% in both cases; leading to the conclusion that the established biofilm is capable of oxidizing the intermediate products generated during ethanol metabolism.

Fig. 2(d) shows the temporal interrelation between ethanol and acetate concentrations in solution. From the experimental data depicted in Fig. 2(d), it is evident that the oxidation of ethanol by SRB proceeded via acetate and that the sole utilization of acetate by acetotrophic bacteria begins upon completion of ethanol degradation, when pH is near 7, and is completed after approximately 4 days from the feeding of the reactor.

The data depicted in Fig. 2(b) show that sulphate are reduced by 85–90% although there is no residual TOC in the solution at the end of the experimental cycle. Similar results have been published by de Smul et al. for an ethanol-fed EGSB reactor operating at near stoichiometry at HRT of 3 h [23] whereas Kaksonen et al. showed that the stoichiometric ratio was adequate to attain around 80% of sulphate reduction with an initial sulphate concentration of 2000 mg/L in a fluidized-bed reactor running at HRT of 20 h [25].

Therefore, to calculate the minimum required organic supplement for the reduction of a given sulphate content, a slight excess above the stoichiometric ratio (reactions (1) and (2)) is required, since a certain amount of the energy produced during the organic material degradation is utilized in bacterial growth and maintenance processes [2]. This amount varies depending on the composition of the sulphate-reducing community, i.e. the different species it consists of and their specific metabolic properties, as well as the studied carbon/energy source. Nagpal et al. determined that the biomass yield of a mixed SRB culture containing the species *Desulfovibrio desulfuricans*, grown on ethanol, varied between 0.75 and 0.93 g DW/mole ethanol [19]. Laanbroek et al.

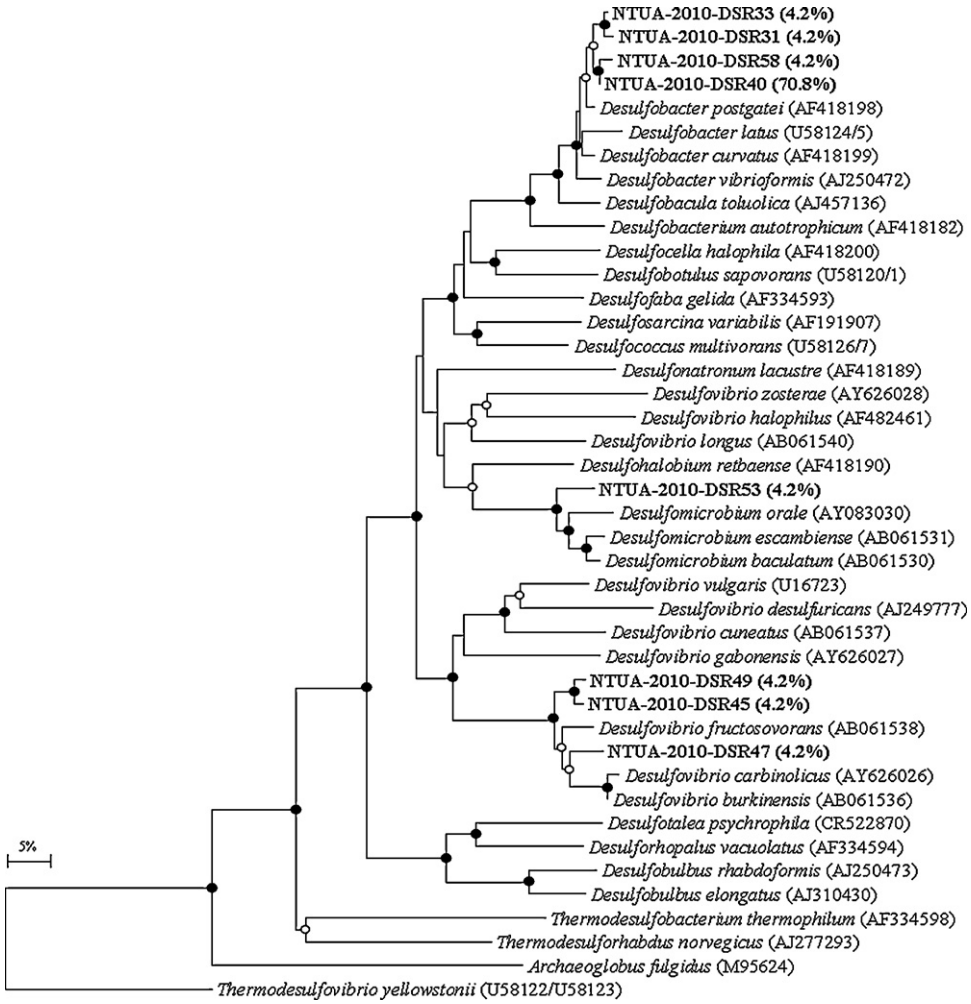

Fig. 4. Neighbor-joining phylogenetic tree showing the relationships between partial *dsr*AB protein (481-residue) sequences retrieved from the column and *dsr*AB sequences from reference SRB strains. Sequences retrieved in this study are in bold, and percentages indicated brackets correspond to the percentages retrieved in the clone library. Circles at the branch nodes represent bootstrap values for 1000 replicates: black circles, 80–100%; open circles, 50–80%. Scale bar, 5 mutation per 100 residues.

showed that the yield coefficient for a pure culture of *Desulfobacter postgatei* grown on a medium containing ethanol and sulphate at a ratio of 0.5, at pH 7.0 and a temperature of 30 °C, was 3 g cell carbon/mole ethanol [44]. This experimental value coincides with the theoretically calculated yield coefficient for the complete oxidation of ethanol by SRB, based on the thermodynamic analysis presented by Rittmann and McCarty [45] and applied by Hatzikioseyian [46].

The mixed culture studied in the present work is largely dominated by *D. postgatei* (Section 3.3). Therefore, according to Laanbroek et al. [44], approximately 12.5% of the added organic material is utilized in bacterial growth processes, resulting in a TOC

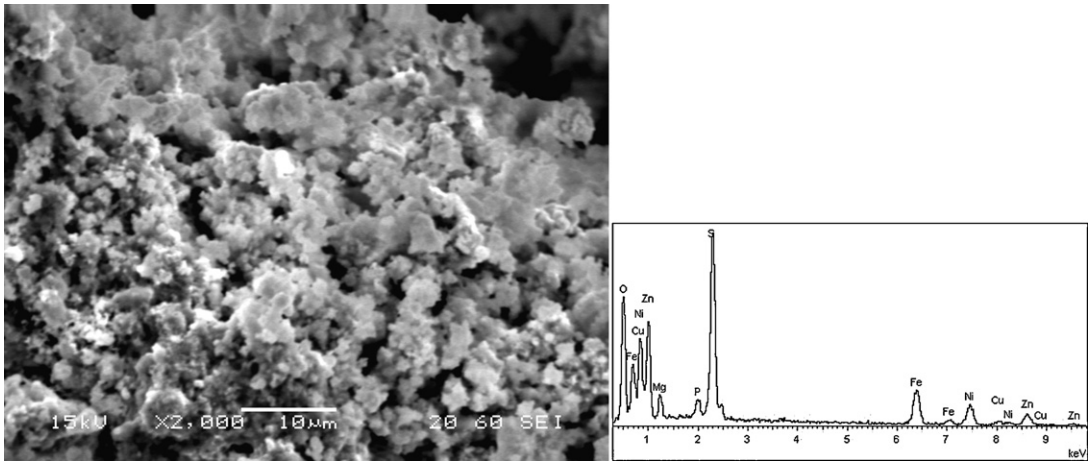

Fig. 5. SEM–EDS analysis on sludge sample collected after the end of the reactor operation: aggregates of microcrystalline iron, zinc, copper and nickel sulphides.

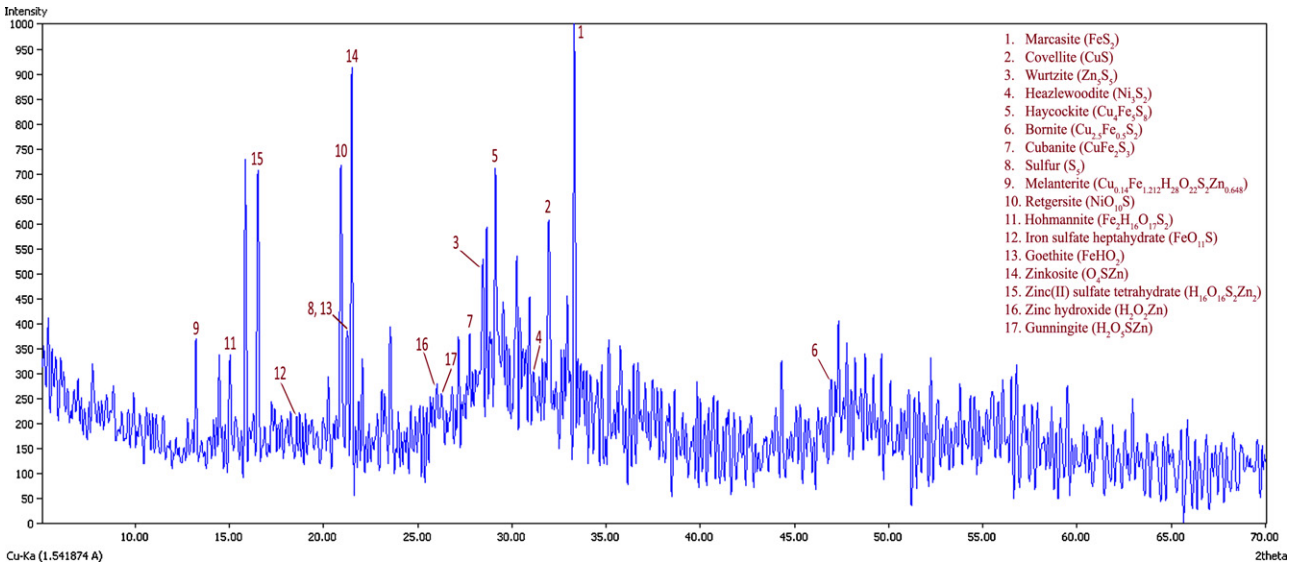

Fig. 6. XRD analysis on sludge sample collected after the end of the reactor operation.

deficit for sulphate reduction which is reflected in the percentage of sulphate that remain in solution at the end of each experimental cycle. This rationale leads to the calculation of a corrected molar ethanol/sulphate ratio to attain complete ethanol oxidation and sulphate reduction in the process presented in this study. This revised stoichiometric ratio is found to be 0.75 and is also confirmed (via the resulting TOC deficit) by our experimental results shown in Fig. 2(b) and (c), where sulphate are reduced by 85–90% and no residual TOC is determined at the end of each experimental cycle.

3.3. Bacterial population

The CE-SSCP molecular fingerprints of 16S rRNA genes (Fig. 3(a)) depict the development of a diverse bacterial community. The results acquired for a sample collected in duplicate at the reactor outlet gave almost identical fingerprints showing high reproducibility. This bacterial community is less diverse than the community originating from the lactate-fed reactor (Fig. 3(b), [34]) which was used as the inoculum for the ethanol-fed reactor presented in this paper. This result shows that the use of ethanol as carbon/electron source limited the growth of certain species present in the inoculum, maintaining a less diverse sulphate-reducing community.

The diversity of this sulphate-reducing community (Fig. 4) was specifically recovered by targeting *dsr*AB genes encoding the dissimilatory sulphite reductase, a key enzyme involved in dissimilatory sulphate-reduction. A diverse population of δ-*Proteobacteria* SRB affiliated to three distinct genera, based on *dsr*AB amino acid sequence comparison, colonized the system. The library was largely dominated (83% of the clones) by *dsr*AB sequences closely related (92–94% similarities) to that of *D. postgatei*. Two *dsr*AB sequences within the *Desulfovibrio* genus (showing 87–91% similarities with *D. fructosovorans*, 84–88% with *D. carbinolicus* and *D. burkinensis*) as well as a *dsr*AB sequence within the *Desulfomicrobium* genus were also found in the column, representing, respectively, 13% and 4% of the clones. Species of all these three genera were also identified in the original lactate-grown culture [34], which was used as the inoculum for the reactor discussed in this paper.

Concerning their ability to completely oxidize ethanol, all species belonging to *Desulfovibrio* and *Desulfomicrobium* genera are unable to metabolize acetate [2,47]. This has been reported specifically for *D. fructosovorans* [48] as well as *D. carbinolicus* and *D. burkinensis* [49]. Furthermore, *D. postgatei*, which degrades acetate [50,51] as all other species belonging to the *Desulfobacter* genus [2], has demonstrated higher specific growth rate (in relation to *Desulfovibrio baculatus* and *Desulfobulbus propionicus*) in the presence of excess ethanol and sulphate [44]. Therefore, the substrate change, from lactate to ethanol, may have favoured the growth of this specific species which, also due to its ability to completely oxidize ethanol, finally dominated the sulphate-reducing community in the reactor, resulting in minimum residual TOC at the end of each experimental cycle (Fig. 2(c)).

3.4. Metal removal and sulphide precipitation

The reactor operated with synthetic metal-bearing solutions containing Fe, Zn, Cu and Ni at initial concentrations of 100 mg/L and 200 mg/L each. All metals precipitated quantitatively within the fist 24 h of each experimental cycle at both concentration levels (Fig. 2(e)). This result points out that the metal-precipitating capacity of the column was maintained high despite the presence of a less diverse sulphate-reducing community (Section 3.3 and Fig. 3).

The elemental composition analysis revealed that the dry sludge contained 5.55% Fe, 8.51% Zn, 1.89% Cu and 10.36% Ni; i.e. metals were 26.31% of dry sample weight, which coincides with the total metal content of the sludge produced from the previously operated sulphate-reducing bioreactor [34]. Volatile suspended solids were determined at 20% of dry sample weight. The remaining content of sludge is attributed to sulphur, bonded to metals and/or elemental.

SEM–EDS analysis revealed aggregates of microcrystalline iron–zinc–copper–nickel sulphides (Fig. 5). The biogenic formation of microcrystalline sulphides has already been discussed in detail in [34].

XRD analysis (Fig. 6) of the precipitates demonstrated the presence of marcasite (2.6894 Å peak at 33.32°) as the main iron sulphide, covellite (2.8009 Å peak at 31.95°) as the main copper sulphide, wurtzite (3.1579 Å peak at 28.26°) as the main zinc sulphide and heazlewoodite (2.8715 Å peak at 31.15°) as the main nickel sulphide. All these biogenically produced minerals, along with some mixed metal sulphides (i.e. haycockite, bornite and cubanite) were identified in the 25–35° range, where the X-ray diffraction analysis revealed a broad diffraction band, confirming the presence of amorphous phases. Marcasite and wurtzite were also the dominant iron and zinc sulphides, respectively, in the sludge produced during

the operation of the lactate-fed reactor presented in [34,35]. Poorly crystalline covellite, biologically produced at ambient temperature, was also identified in the precipitates of sulphate-reducing processes [52,53]. The formation of heazlewoodite is consistent with the findings of Gramp et al. [54] in biogenic sulphide precipitates.

4. Conclusions

The following conclusions can be drawn from this study:

1. The lactate-grown sulphate-reducing culture acclimated successfully to the change of carbon/electron source, depicting a possible adaptability advantage of mixed cultures over pure ones.
2. The substrate change favoured the growth of the acetate-utilizing species, resulting in complete oxidation of the intermediately, microbially produced acetate and, thus, minimum residual organic content in solution after each experimental cycle. This was mainly attributed to the presence of *D. postgatei* species which dominated the sulphate-reducing community in the reactor.
3. All metals precipitated quantitatively within the fist 24 h of each experimental cycle up to 200 mg/L. The metal-precipitating capacity of the column was maintained high despite the presence of a less diverse sulphate-reducing community.
4. The precipitated metal sulphides were mainly microcrystalline and included marcasite, covellite, wurtzite and heazlewoodite.
5. At the stoichiometric ethanol/sulphate ratio, sulphate reduction proceeded up to 85–90%. The minimum required organic supplement for the reduction of a given sulphate content should be at a slight excess above the stoichiometric ratio, since a certain amount of the energy produced during the organic material degradation is utilized in bacterial growth and maintenance processes.
6. Assuming that 12.5% of the added organic material is required for the growth of *D. postgatei* on ethanol, as suggested in the literature, the experimentally observed TOC deficit for sulphate reduction can be sufficiently explained. This led to the calculation of the required molar ethanol/sulphate ratio to attain complete ethanol oxidation and sulphate reduction in the process.

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