The preferential localisation of SRB in an acetate supplemented up-flow anaerobic packed bed reactor

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Abstract

Biological sulphate reduction (BSR), catalysed by consortia of sulphate reducing bacteria (SRB) represents a low-cost and sustainable remediation strategy for low-flow acid mine drainage (AMD) effluents. This study investigates the performance and the microbial ecology throughout an acetate-supplemented up-flow anaerobic packed bed reactor (UAPBR). The reactor, operated at a four-day hydraulic retention time (HRT), achieved 78% sulphate removal in the first third of the reactor, and 97% removal in the effluent. Metagenomic 16S rRNA gene sequencing identified eight SRB operational taxonomic units (OTUs) which were preferentially located between the planktonic and biofilm communities as well as different zones along the reactor.

Keywords: Biological sulphate reduction (BSR), sulphate reducing bacteria (SRB), 16S rRNA metagenomics, biofilm.

Introduction

Acid mine drainage (AMD) is a serious form of pollution in countries with extensive mining operations, characterised by acidified water with high concentrations of sulphate and dissolved heavy metals (Johnson and Hallberg 2005). AMD originating from diffuse sources, such as abandoned mines and tailing impoundments often generate smaller volumes of AMD, but the number of these sites and longevity of the generation has created a problem requiring low-cost remediation strategies which can operate sustainably for decades to come.

Biological sulphate reduction (BSR) has been demonstrated at laboratory- and pilot-scale, as an effective low-cost strategy for the remediation of low-flow AMD (Kolmert and Johnson, 2001; Lens et al., 2002; Boshoff et al., 2004; Zagury and Neculita, 2007). This process uses mixed consortia of sulphate reducing bacteria (SRB), which use sulphate as a terminal electron acceptor in the oxidation of organic compounds, resulting in the formation of sulphide and carbonate (Muyzer and Stams 2008). The sulphide product can be used to precipitate contaminant heavy metals, or be biologically oxidised to sulphur as a value-added product, and the carbonate aids in neutralisation of the acidic solution, making this process ideal for the treatment of AMD effluents.

Implemented BSR systems must overcome two major issues associated with this process: the low bacterial growth rates associated with SRB; and the provision of a suitable low-cost electron donor. The low growth rates of SRB can be overcome by decoupling the hydraulic and biomass retention times within BSR reactor systems. This has been achieved through granulation of SRB biomass in fluidized bed reactors (Alphenaar et al., 1993), or through providing a large surface for microbial attachment and biofilm formation within the reactor (Bachmann et al. 1985; Zhang and Wang 2016; Hessler et al. under review). Acetate is a low-cost and widely available electron donor with potential to be generated from waste streams such as anaerobic digestion. However, acetate supports considerably lower SRB growth rates than other more expensive electron donors such as ethanol and lactate (Thauer et al. 1977). Efficient sulphate removal using acetate therefore requires significant

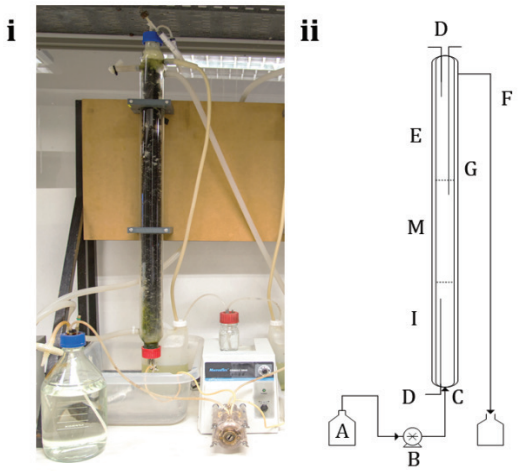

Figure 1 *Photograph* ***(i)*** *and annotated schematic diagram* ***(ii)*** *of the UAPBR used in this study. The feed solution (A) was continuously supplied to the reactor via a peristaltic pump (B) via the inlet (C) at the base of the reactor. The reactor is demarcated into three sequential zones, namely the inlet (I), middle (M) and effluent zone (E). Sampling ports (D) at the base and top of the reactor are used to sample for solution chemistry leaving each zone. Effluent is discharged by gravity via an overflow tube (F). The reactor was maintained at 30˚C by a circulating waterbath and glass heating jacket (G).*

biomass accumulation (Harada 1994).

Previous BSR reactor studies have described the ecology of these systems but have typically characterised a single sample, assuming microbial homogeneity throughout the reactor. Differences in the speciation between planktonic cells and those associated with biofilms have been described in bioleaching (Wang et al. 2014) and marine environments (Rickard et al. 2003), but have not yet been investigated within BSR systems. This study looks to describe the reactor performance and the detailed ecology of an acetate-supplemented Up-flow anaerobic packed bed reactor (UAPBR), characterised by plug-flow fluid dynamics. The SRB ecology of the planktonic and biofilm communities, from multiple reactor zones, are analysed by 16S rRNA sequencing of extracted metagenomic DNA.

Methods

Reactor system and operation

This study was conducted using a 1 L glass up-flow anaerobic packed bed reactor with an internal diameter of 4 cm and height of 80 cm (fig. 1) as described in Hessler et al (under review). The reactor was packed with open-pore polyurethane foam cubes of approximately 2 cm^3. The reactor was inoculated with a composite culture drawn together from a number of long-term SRB stock reactors. The reactor was operated at a four-day HRT and fed sterile modified Postgate B media (Postgate 1984) containing 10.4 mM sulphate, supplemented with 11.2 mM sodium acetate at neutral pH.

Analytical methods

The bulk liquid leaving each of the three reactor zones was sampled for solution chemistry by drawing 2 ml via the reactor sampling ports (Fig.1). The residual sulphate and the produced sulphide concentrations were determined by the APHA 1975 turbimetric method (Greenberg and Eaton 1999) and DMPD method described by Cline (1969), respectively. The concentration of acetate was determined by high performance liquid chromatography (HPLC) using a Waters Breeze 2 system equipped with a Bio-Rad Organic Acids ROA column and UV (210 nm wavelength) detector. The system was operated with a 0.01M H_2SO_4 mobile phase, with a flow rate of 0.6 ml/min.

Steady-state biological sampling

Steady-state conditions were assumed to be established within the UAPBR when residual sulphate concentrations leaving each reactor zone varied by less than 10% over a minimum period of three hydraulic residence times. Each zone of the reactor was sampled a total of eight times for solution chemistry and once for biological material during the defined steady state period. The cells attached and weakly associated to the polyurethane foam matrix, present in the inlet and effluent zones, were isolated using a modified detachment protocol and together with the planktonic cells present in the bulk liquid of each of the three zones, quantified by direct cell counting (Hessler et al. 2017).

The planktonic community was harvested from the reactor by removing 15 ml of bulk liquid, via the respective sampling ports, followed by centrifugation (10 000 g for 10 min at room temperature) to recover the microbial cells. The genomic DNA was immediately extracted from the resulting cell pellets, as described below. Matrix attached and associated cells were recovered from the UAPBR by aseptically removing polyurethane foam pieces from the inlet and effluent regions. The matrix associated cells were removed from the foam by mild agitation in reactor media, followed by centrifugation (10 000 g for 10 min at room temperature). The total genomic DNA of the matrix attached communities were then extracted directly off the polyurethane foam. Scanning electron microscopy (SEM) was used as a visual conformation of the microbial colonisation of the polyurethane foam. Polyurethane foam was removed from the inlet zone and prepared for SEM as described in Hessler et al. (2017) and viewed using a FEI NOVA NANO SEM 230.

DNA extraction and Sequencing

Total genomic DNA was extracted and purified from the cell pellets and polyurethane foam, collected as described above, using a NucleoSpin® Soil Genomic DNA Extraction Kit (Machery-Nagel, Germany) according to the manufacturer's instructions. The purified genomic DNA was subsequently sent to Macrogen Korea for sample preparation, Illumina® MiSeq® sequencing, read pre-processing, clustering and taxonomic assignment. Briefly, the bacterial V3-V4 region of the 16S rRNA gene was PCR amplified using dual-index barcoded primers FwOvAd_341F and ReOvAd_785R. Fast Length Adjustment of Short reads (FLASH; Magoč and Salzberg 2011) was used to merge the paired-end reads. Read trimming, filtering and OTU picking was performed using CD-HIT-OTU (Li et al. 2012). The taxonomy of the OTUs was then assigned against the RDP 16S rRNA classifier algorithm (Edgar 2010) using QIIME, UCLUST (Langille et al. 2013).

Results

Reactor performance

The UAPBR showed effective sulphate conversions at a four-day HRT, with 78% removal within the inlet zone of the reactor (Fig. 2B), corresponding to a volumetric sulphate reduction rate (VSRR) of 0.26 mmol/L.h. The concentration of acetate leaving the inlet zone of 11.6 mM is greater than predicted based on the equimolar oxidation of acetate coupled to the reduction of sulphate. The excess acetate is likely the result of the oxidation of other Postgate B media components, namely citrate and yeast extract. Subsequent sulphate scavenging was seen in the middle and effluent reactor zones, bringing the total sulphate removal to 97%, with an overall VSRR of 0.11 mmol/L.h. The 1.9 mM sulphate removed in the middle and effluent zones corresponded with an approximately 2-fold equimolar reduction in the acetate concentration, indicating that acetate oxidation, by SRB and non-sulphate reducing microorganisms, had taken place.

Biomass retention

The UAPBR successfully developed an attached biofilm community on the incorporated polyurethane foam (Fig 2D). Quantification of the attached cells within the inlet zone revealed that this community was two to three orders of magnitude more concentrated than the planktonic cells which remained constant throughout the three zones at approximately $2 \times 10^8 \pm 9.3 \times 10^7$ cells/ml (Fig. 2C). The concentration of attached cells within the effluent zone was lower, at $1.1 \times 10^8 \pm 2.1 \times 10^7$ cells/ml.

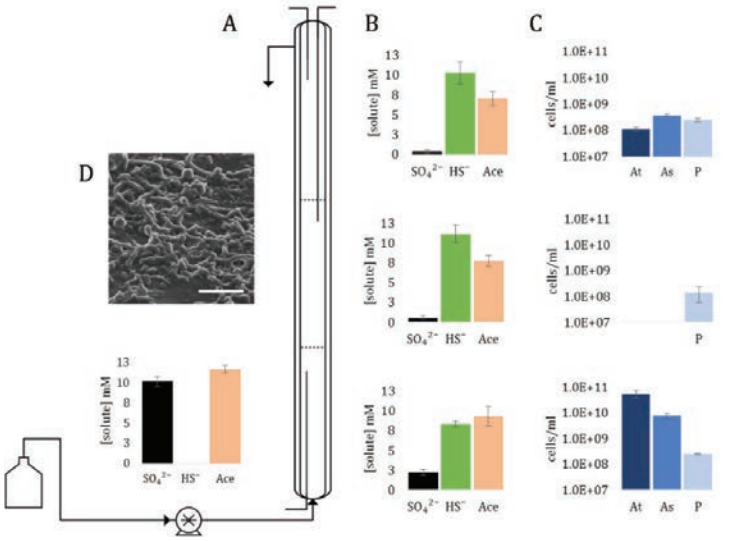

Figure 2 *Schematic diagram of the UAPBR used in this study* ***(A)*** *showing the three sequential reactor zones. The sulphate, sulphide and acetate concentration in the feed and leaving each of these zones* ***(B)*** *is shown. The concentration of planktonic (P) cells, cells attached to (At) and weakly associated (As) with the polyurethane foam* ***(C)*** *were determined by detachment protocol and direct cell counting. The attached and associated cell concentration of the middle zone was not determined due inaccessibility to this zone. Error bars represent one standard deviation of the mean. SEM image* ***(D)*** *of colonised polyurethane foam from the inlet region of the UAPBR. Scale bar represents 4 μm.*

Bacterial community structure

The planktonic microbial communities from each of the three zones as well as attached and associated cells from the inlet and effluent zones were resolved using 16S rRNA gene metagenomic sequencing. The overall community structure at the phylum level was similar between all samples, and predominantly consisted of Proteobacteria and Bacteriodetes (Fig. 3A). A number of other bacterial phyla, including Firmicutes, Synergistetes, Thermotogae and Verrucomicrobia, made up the remaining 14 – 27% of these microbial communities. Thermotogae and Verrucomicrobia showed an inverse propensity for planktonic versus biofilm communities.

The OTUs that could be identified as SRB were classified within the Proteobacteria class of Deltaproteobacteria. These Eight SRB OTUs belong to six genera, namely *Desulfomicrobium, Desulfovibrio, Desulfobacter, Desulfarculus, Desulfatitalea* and *Desulfobulbus* (Fig. 3B). The absolute cell concentrations of these OTUs were calculated by multiplying their relative abundance by the determined total cell concentration of each community. These SRB OTUs showed significant distinctions between the planktonic and biofilm communities. A *Desulfomicrobium* and *Desulfovibrio* OTUs made up over 90% of the SRB OTUs present within the planktonic communities in each of the three reactor zones. However, these SRB made up less than 10% of the attached community within the same zones. The inlet attached and associated SRB community was dominated by *Desulfobulbus, Desulfarculus* and *Desulfobacter.* The attached community within the effluent zone contained very low numbers of the *Desulfomicrobium* and *Desulfovibrio* OTUs present in the planktonic community, instead made up predominantly the same *Desulfobulbus* OTU identified in the inlet zone. Two *Desulfatitalea* OTUs were found almost exclusively in the attached and associated communities in the inlet and effluent zones but made up a greater proportion of the community within the effluent zone. These three microorganisms, being present in this zone, are likely suited to sulphate and acetate scavenging and can tolerant high concentrations of sulphide.

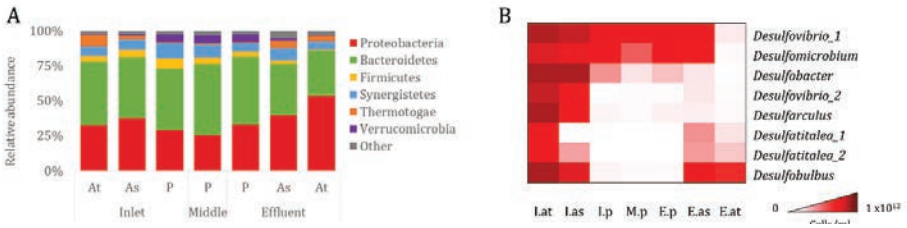

Figure 3 *The bacterial community structure, at the phylum level* ***(A)****, of the planktonic (P), associated (As) and attached (At) microbial communities from the inlet, middle and effluent zones of the UAPBR. The absolute cell concentrations of the eight identified SRB OTUs* ***(B)*** *present in the planktonic (p), associated (As) and attached (At) communities of the inlet (I), middle (M) and effluent (E) zones of the UAPBR are shown in a heatmap.*

Conclusion

The acetate supplemented UAPBR showed effective sulphate conversion and VSRRs at a four-day HRT. Acetate represents one of the most viable electron donors for use in passive and semi-passive bioremediation of AMD, due to its low cost and wide availability. The incorporation of a biomass support matrix, polyurethane foam, into the UAPBR allowed for the decoupling of the biomass and hydraulic retention time, successfully addressing the low bacterial growth rates on acetate and enhancing biomass retention within the continuous reactor system. The SRB within the inlet's attached and associated communities are likely responsible for most of the sulphate reduction within the system.

Metagenomic 16S rRNA gene sequencing of the bacterial communities revealed differing prevalence of SRB OTUs between biofilm and planktonic communities as well as across the different zones of the reactor. *Desulfobulbus, Desulfarculus* and *Desulfobacter* were the dominant SRB attached and associated to the polyurethane foam within the inlet zone of the reactor. In contrast the SRB community within the planktonic phase throughout the reactor were dominated by *Desulfomicrobium* and *Desulfovibrio*. The attached and associated SRB community within the effluent zone of the reactor were colonised predominantly by a *Desulfobulbus* OTU, likely responsible for the sulphate scavenging in this zone. It is important to note the limitation of 16S rRNA gene metagenomic sequencing for the identification of microorganisms on the basis of their metabolic function. It is possible that microorganisms other than those identified above may be contributing to sulphate reduction. This approach combined with further functional gene studies or whole genome sequencing will enable a more comprehensive description of the sulphate reducing potential of BSR reactor communities.

However, the differing SRB ecology identified between the inlet zone, responsible for rapid acetate oxidation and sulphate reduction, and the effluent zone, responsible for sulphate scavenging, provides support for a zoned BSR reactor configuration. The incorporation of polyurethane supports allows not only for biomass retention but the retention of several SRB genera found in low abundance in the planktonic communities. This confers the reactor with increased SRB diversity and potentially improved system robustness. The physiochemical conditions needed to stimulate attachment and biofilm formation of these SRB onto solid supports warrants further investigation.

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