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Microbial generation of sulfuric acid from granular elemental sulfur in laboratory scale bioreactors

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Abstract

The EU Horizon 2020 project “BioMOre” aims to develop a deep *in situ* biomining biotechnology, which would be a promising alternative to conventional mining operations, recovering metals from sulfidic ores buried deep in Earth’s crust economically, while minimizing impacts on the environment. The concept involves sequential acid leaching (to dissolve acid-labile minerals) and indirect bioleaching to extract and recover base metals from deep-buried ores. Sufficient provision of sulfuric acid at the mine site is essential for the acid leaching stage. A prototype bioreactor at the mine site generating sulfuric acid from elemental sulfur which is a relatively cheap, available, and safe material, as an alternative to using chemically-produced sulfuric acid, would eliminate transporting this hazardous material, and would highlight further the green credentials of the deep *in situ* biomining technology. Two laboratory-scale, sulfuric acid-generating bioreactors (SAGBs), in which sulfuric acid was microbiologically generated from elemental sulfur at two different temperatures, were commissioned. The system ran at 30 °C was dominated by sulfur-oxidizing mesophiles (*Acidithiobacillus albertensis, Sulfobacillus thermosulfidooxidans*, and a novel *Acidibacillus* sp.), and the one at 50 °C by a moderately thermophilic bacterium (*Acidithiobacillus caldus*) and a thermophilic archaeon (*Sulfolobus metallicus*). Different conditions were tested to optimize the biological production of sulfuric acid (pH 0.8 and 1.0). Higher sulfate production was not achieved, but effluent acidity was successfully augmented by addition of magnesium sulfate (corresponding to 200 mM

sulfate) in the feed of the SAGB ran at 30 °C, due to increased buffering afforded by the sulfate/bisulfate couple.

1 Introduction

Biomining, the use of microorganisms to recover metals by oxidative dissolution of sulfidic minerals in ores, has developed into an economical biotechnology (Rawlings and Johnson, 2007), which is conventionally carried out in dumps, heaps or stirred tanks (Brierley, 2008). *In situ* biomining represents an alternative approach, which is currently used to extract uranium from shallow aquifers (Mudd, 2001). The concept of the European Union Horizon 2020 project “BioMOre” (www.biomore.info), combining “deep *in situ* biomining” (Johnson, 2015) and indirect bioleaching (Schippers and Sand, 1999; Rawlings, 2004), has been described in more detail by Pakostova et al. (2016), while Johnson (2015) summarized the numerous advantages of this approach. The Kupferschiefer copper black shale ore deposit in Rudna (Poland) was selected as a test site for BioMOre. Kupferschiefer deposits, spreading across Germany and Poland, are Europe’s largest copper reserve, and they have been exploited for years. They are calciferous, carbon rich marly clays, containing finely dispersed sulfidic copper-bearing minerals (mainly chalcocite, bornite, and chalcopyrite), and other valuable elements (Ag, Pb, Zn in larger amounts) (Kutschke et al., 2015). The BioMOre concept involves drilling down into, and fracturing, the ore body, and injecting an acidic liquor to dissolve present basic minerals and solubilize acid-labile metal sulfides. Indirect dissolution of the sulfide minerals by an acidic, ferric iron-rich lixiviant, generated in bioreactors (e.g. Livesey-Goldblatt et al., 1977) will follow. Copper will be solubilized and extracted from the pregnant leach solution (PLS). Ferrous iron in the raffinate solution will then be re-oxidized in the bioreactor, and the regenerated lixiviant recirculated into the ore body.

In this study, a laboratory tests to optimize production of sulfuric acid, which could be used for basic mineral dissolution prior to indirect leaching from metal-bearing sulfide ores, in continuous-flow bioreactors were carried out. Even though it is not envisaged that a sulfuric acid-generating bioreactor (SAGB) will be used at the BioMOre mine site as there is sufficient provision of sulfuric acid, developing a prototype SAGB could be appropriate to other sites. Sulfuric acid can be generated from elemental sulfur (S^0), an available and safe material produced as a byproduct of removing sulfur-containing

contaminants from natural gas, petroleum (Dehghani and Bridjanian, 2010) and coal (Ambedkar et al., 2011), and hazardous acid transport could be eliminated. Oxidation of S^0 by extremely acidophilic sulfur-oxidizing bacteria and archaea (defined as having pH optima for growth of ≤ 3), which have recently been reviewed by Dopson and Johnson (2012), generates sulfuric acid, according to the following reaction:

$$2\ S^0 + 2\ H_2O + 3\ O_2 \rightarrow 3\ H^+ + HSO_4^- + SO_4^{2-}$$

The ability of iron-and sulfur-oxidizing microorganisms to generate ferric iron and/or sulfuric acid is used in biomining to create conditions that cause oxidative dissolution of sulfides. Currently, metals (including copper, cobalt, nickel, uranium and gold) are recovered from primary ores and mine wastes in full-scale commercial operations.

2 Material and Methods

2.1 Sulfuric acid-generating bioreactor at 30 °C (30SAGB)

A laboratory-scale reactor was commissioned to generate biogenic sulfuric acid (30SAGB). 2 L glass reactor vessel fitted with stainless steel top plates and various inserts (Electrolab, U.K.) was filled with 2 L of a solution containing basal salts and trace elements (Ňancucheo et al., 2016), adjusted to pH 2.0 with sulfuric acid. The reactor vessel was sterilized by autoclaving. After cooling, 800 g of granular sulfur (Peak Trading Company, Nottingham, U.K., purity ≥ 99.99%) (> 2 mm diameter) was added to the vessel. The reactor was then inoculated with 4 strains of acidophilic, sulfur-oxidizing bacteria and archaea, maintained in the Acidophile Culture Collection at Bangor University, UK (*Acidithiobacillus (At.) albertensis*^{T}*, At. caldus*^{T}*, Sulfobacillus (Sb.) thermosulfidooxidans*^{T}, and *Sulfolobus (S.) metallicus*^{T}). The prokaryotes and their properties are summarized in Table 1. The temperature of the 30SAGB was maintained at 30 °C and the bioreactor was aerated (at ~ 1 L/min) with filter-sterilised air. Sulfuric acid was microbially generated, and when the pH reached 1.0 (in 11 days), the bioreactor was connected to an influent liquor described above, but this time adjusted to pH 3.0. The bioreactor pH was maintained via automated addition of this less acidic medium into the bioreactor (FerMac 260 pH control, Electrolab, U.K.). Ecoline Ismatec peristaltic pump (Bennett Scientific Ltd., UK) was used to remove the biogenic sulfuric acid from the culture vessel. For 10 days, effluent volumes were daily measured (and withdrawn), and analyzed for sulfate concentrations and planktonic cells numbers. The bioreactor pH setting was changed to 0.8, and after reaching the

value (4 days) the same parameters were monitored for 14 days. The influent liquor was amended with 200 mM magnesium sulfate (in form of $MgSO_4 \times 7H_2O$), and the effluent liquors were monitored for 12 days. Finally, pH 1.0 sulfuric acid generation in presence of 200 mM extra sulfate was analyzed for 9 days.

2.2 Sulfuric acid-generating bioreactor at 50 °C (50SAGB)

A second bioreactor (50SAGB) was commissioned. The same procedure was followed, except that 690 g of granular sulfur was used, and the bioreactor was inoculated with the thermotolerant bacterium *At. caldus*^{T} and thermophilic archaeon *S. metallicus*^{T} (Table 1). The temperature of the 50SAGB was maintained at 50ºC using a FerMac 240 temperature controller (Electrolab). First, a pH 1.0 sulfuric acid generation was tested for 9 days. Then the pH in the bioreactor was let to drop to 0.8, which took 5 days. The generation of a pH 0.8 sulfuric acid was monitored for 12 days, after which the last phase, sulfuric acid (pH 0.8) in the presence of extra 200 mM magnesium sulfate in inlet medium, ensued for 22 days.

2.3 Chemical analyses

The pH values of SAGBs effluent liquors were offline measured using a pHase combination glass electrode (VWR International, UK), coupled to an Accumet pH/redox meter 50. Filtered influent and effluent samples were analyzed for concentrations of sulfate, using a Dionex IC25 ion chromatograph with an Ion Pac AS-11 column equipped with a conductivity detector. Concentrations of microbially generated sulfate [SO_4] were calculated from effluent [$SO_{4\ effluent}$] and influent sulfate concentrations [$SO_{4\ influent}$], flow rates (F) and the bioreactor effective volumes ($V_{E\ 30SAGB}$ = 1.97 L; $V_{E\ 50SAGB}$ = 1.92 L), according to the following equation:

$[SO_{4\ generated}]$ (moles/h/L) = $(\Delta SO_4 \times F) / V_E$

where: $\Delta SO_4 = [SO_{4\ effluent}] - [SO_{4\ influent}]$

Sulfuric acid is a diprotic acid, and therefore dissociates in two steps. As a strong acid, it completely dissociates in aqueous solutions to form hydronium ions (H_3O^+) and hydrogen sulfate (HSO_4^-). The conjugate base of sulfuric acid, also called the bisulfate ion (HSO_4^-), dissociates in dilute solutions, forming more hydronium ions and sulfate ions (SO_4^{2-}):

$H_2SO_4 \rightarrow H^+ + HSO_4^-$, $pK_{a1} = -3$

$HSO_4^- \rightarrow H^+ + SO_4^{2-}$, $pK_{a2} = 1.99$

According to the Henderson-Hasselbach equation (below), bisulfate ions constitute 90.7% of the analyzed total sulfate concentrations at pH 1.0, and 93.9% at pH 0.8.

$pH = pK_a + \log [SO_4^{2-}] / [HSO_4^-]$

The total acidity of the SAGB effluents consists of two components (concentration of protons $[H^+]$ and bisulfate ions $[HSO_4^-]$), and should therefore be increased by additional sulfate present in the influent media, as extra protons could be released from bisulfate ions. Generated acidity was calculated from pH values and sulfate data according to the following equation:

$[\text{acidity}_{\text{generated}}]$ (moles/h/L) = (Δ acidity × F) / V_E
where: Δ acidity = $[H^+_{\text{effluent}}] + [HSO_4^-{}_{\text{effluent}}] - [H^+_{\text{influent}}] - [HSO_4^-{}_{\text{influent}}] = [10^{-\text{pH effluent}}] + p_1 \times [SO_{4\ \text{effluent}}] - [10^{-3}] - 0.089 \times [SO_{4\ \text{influent}}]$

The p_1 coefficient describes the dissociation of bisulfate, and equals 0.907 and 0.939 for pH 1.0 and pH 0.8 effluent, respectively.

SAGB effluents were analyzed for the presence of sulfur intermediates during each tested phase. Concentrations of tetrathionate and thiosulfate were determined using a colorimetric assay (Sörbo, 1957), as modified by Kelly et al. (1969). To detect colloidal sulfur which could serve as a substrate for the acidophiles, 100 mL granular sulfur-free samples were aseptically withdrawn from each bioreactor, and incubated aerobically in shaken 250 mL conical flasks at relevant temperatures (30 or 50 °C). After a week, pH, cell counts and sulfate concentrations were determined, and compared to the initial values.

2.4 Microbiological and biomolecular analyses

Planktonic microorganisms were enumerated using a Thoma counting chamber and a Leitz Wetzlar 766200 (Germany) phase contrast microscope, at ×400 magnification. Planktonic microbial populations were analyzed on last day of each tested condition (pH = 1.0, pH = 0.8, pH = 0.8 with additional sulfate, pH = 1.0 with additional sulfate) by terminal restriction enzyme fragment length polymorphism (T-RFLP), using protocols described elsewhere (Kay et al., 2013). Two restriction enzymes were used to analyze both bacterial (HaeIII and CfoI), and archaeal (CfoI and AluI) populations.

The relative abundance of acidophilic prokaryotes in 50SAGB was determined on the basis of cellular morphologies.

3 Results

3.1 Sulfuric acid-generating bioreactor at 30 °C (30SAGB)

Determined flow rates reflected sulfuric acid production rates in the bioreactor. As expected, effluents of pH 1.0 were generated faster compared to pH 0.8 sulfuric acid (Fig. 1a), and the course of planktonic cell numbers in the effluents was inversely proportional to flow rates (Fig. 1b). Sulfate production (Fig. 1c) was similar in pH 1.0 and 0.8 effluents, after disregarding the first value of the first two phases, the average values equaled to 0.71 and 0.72 mmol/h/L, respectively. When medium amended with additional sulfate was used to microbiologically generate pH 0.8 sulfuric acid, the average sulfate production dropped to 0.31 mmol/h/L. The highest sulfate production was achieved while pH 1.0 sulfuric acid was generated from a medium amended with 200 mM sulfate, its average value reaching 0.89 mmol/h/L. With non-supplemented media, the total generated acidities (Fig. 1d) were again comparable, accounting on average for 1.38 and 1.21 mmol H^+/h/L in pH 1.0 and 0.8 effluents, respectively. In agreement with sulfate production, lowest acidity generated (average value of 0.87 mmol H^+/h/L) was observed in pH 0.8 sulfuric acid produced from a medium amended with magnesium sulfate, while highest acidity (1.47 mmol H^+/h/L) was achieved during generation of pH1.0 sulfuric acid from the amended medium.

Neither tetrathionate nor thiosulfate was detected in any of the effluent liquors. No changes in pH, cell counts or sulfate concentrations in sulfur-free effluent samples were recorded after a week incubation in flasks, indicating no additional microbial growth or oxidation.

An unknown bacterium was isolated from the 30SAGB, identified as a new species of the genus *Acidibacillus* (phylum Firmicutes, order *Bacillales*, family *Alicyclobacillaceae*), based on analysis of 16S rRNA gene sequence. It dominated the bioreactor for the first 13 days after assemblage (analyzed by T-RFLP analysis, data not showed), out of which 11 were before the pH inside the 30SAGB reached 1.0 when the continuous sulfuric acid generation commenced. The bacterium receded thereafter, but its presence was detected over the whole time course of the experiment, its relative abundance ranging from 10 to 19% (Fig. 3). The most

abundant bacterium (accounting on average for 80% of the total bacterial population) in pH 1.0 effluent liquors, regardless whether the inlet medium had or had not been amended with 200 mM sulfate, was *At. albertensis*, while *Sb. thermosulfidooxidans* dominated the pH 0.8 effluents (71%, relative abundance in absence, and 81% in presence of additional sulfate). *At. caldus* accounted for 0–2% of the bacterial populations (Fig. 3). No archaea were detected by T-RFLP analyses (data not shown).

3.2 Sulfuric acid-generating bioreactor at 50 °C (50SAGB)

Flow rate values during the first two phases were comparable, but when medium amended with magnesium sulfate was used, they decreased considerably (Fig. 2a). Planktonic cell numbers did not vary much within the course of the whole experiment (Fig. 2b). Compared to 30SAGB, generation of both sulfate and acidity was significantly lower at 50 °C (Fig. 2c); the average values were 0.27 mmol SO_4^{2-}/h/L and 0.46 mmol H^+/h/L in pH 1.0 effluents, 0.35 mmol SO_4^{2-}/h/L and 0.64 mmol H^+/h/L in pH 0.8 sulfuric acid, and dropped to 0.02 mmol SO_4^{2-}/h/L and 0.31 mmol H^+/h/L in presence of additional sulfate in influent medium. The experiment was terminated after this phase, due to poor performance. No sulfur intermediates were detected in any of 50SAGB effluents.

Both inoculated sulfur-oxidizing acidophilic prokaryotes were detected in all samples by T-RFLP analyses (data not shown). Relative cell counts, on the basis of cellular morphologies, indicated that *At. caldus* was more abundant in the 50SAGB effluents, accounting for 72 to 81% of total microbial populations, than *S. metallicus* (19 to 28% relative abundance) (Fig. 4).

4 Discussion

To minimize loses of substrate to outflow, granular sulfur which stayed by the bottom of the bioreactors was used. Contrary to expectations, the 50SAGB did not generate more sulfate or acidity compared to 30SAGB, and was terminated prematurely. The possible reasons are: (i) 50 °C is not an optimum growth temperature for either *At. caldus* or *S. metalicus* (Table 1). (ii) pH 1.0 is described in literature as a pH minimum for both prokaryotes present in 50SAGB, which means that pH 1.0 was possibly, and pH 0.8 most probably, too low for their optimal activity. Nevertheless, both organisms were present (confirmed by T-RFLP and microscopy) in all three tested types of 50SAGB effluents. Although the volumes of pH 1.0 sulfuric acid (described by flow

rates) generated in both bioreactors were larger compared to pH 0.8 acid generation, the sulfate concentrations analyzed in the effluents were proportionally lower, and increased generation of sulfate was therefore not achieved. However, the total acidity in 30SAGB pH 1.0 effluents was raised by addition of magnesium sulfate to influent medium. This was not achieved in pH 0.8 effluents, probably due to abovementioned lower flow rates.

The sulfur oxidations pathways in acidophiles have been not long ago reviewed by Rohwerder and Sand (2007) and Johnson and Hallberg (2009). Even though the sulfur oxidation pathways in acidophiles have not been fully elucidated yet, several models have been proposed (e.g. Quatrini et al., 2009; Mangold et al., 2011), indicating that thiosulfate and tetrathionate play roles of intermediates in the bacterial process. Nevertheless, neither was detected in any of the effluent liquors, most probably because sulfur utilization takes place in the periplasmic space or/and in the cytoplasm, as suggested by many studies on *Acidithiobacillus* spp. (Meulenberg et al., 1992; Tano et al., 1996; Hallberg et al., 1996; de Jong et al., 1997a,b; Bugaytsova and Lindström, 2004; Janiczek et al., 2007). Moreover, thiosulfate is stable only in neutral or alkaline solutions, but not in acidic solutions, due to decomposition to sulfite and sulfur. No additional microbial growth or oxidation were recorded in sulfur-free effluent samples, indicating absence of other sulfur intermediates which could be oxidized and thus promote growth of the sulfur-oxidizers.

As expected, *At. albertensis* which has the lowest pH optimum (~0.5) from all the prokaryotes used in this study (Table 1), dominated the pH 1.0 30SAGB effluents. What had not been anticipated though was the predominance of *Sb. thermosulfidooxidans* (with pH optimum 1.1) in pH 0.8 liquors. It is possible that in the conditions out of pH and temperature optima of the autotrophic sulfur-oxidizers, organic carbon was more available, due to cell death and lysis, to support the growth of the mixotrophic bacterium. However, the other inoculated mixotroph, *At. caldus*, was detected only in small numbers over the course of the experiment. As the new *Acidibacillus* sp., indigenous on the used granular sulfur, and isolated from 30SAGB, was not detected in the 50SAGB at any point of the experiment, it could be assumed it was a mesophilic species. Preliminary results showed that the novel *Acidibacillus* sp. shared 97% similarity of its 16S rRNA genes with *Acidibacillus (A.) sulfuroxidans*^{T}, which oxidizes elemental sulfur, and 94% with *A. ferrooxidans*^{T}, which does not utilize

reduced sulfur compounds. In agreement with the requirements of the two abovementioned *Acidibacillus* species (characterized by Holanda and co-workers, 2016), the newly isolated bacterium seemed to require a source of organic carbon for growth, which was presumably present as lysates and exudates of the other sulfur-oxidizers. After isolation, yeast extract had to be present to achieve growth, and possibly served as both energy and carbon source. If that case, the novel *Acidibacillus* sp. is along with those characterized by Holanda (2016) a facultative chemolithoheterotroph. *A. sulfuroxidans* showed a pH optimum and minimum for growth of 1.8 and 1.6, respectively, while corresponding values for *A. ferrooxidans* were 2.9 and 1.9 (Holanda et al., 2016). A pH optimum of the novel bacterium similar to those mentioned above would explain while it dominated the 30SAGB at the beginning, when the pH was decreasing from pH 2.0, but receded shortly after reaching pH 1.0.

5 Conclusion

A new concept combining “deep *in situ* biomining” and indirect bioleaching is being developed within the BioMOre project. An initial leaching with acidic liquor to dissolve present basic minerals enables *in situ* leaching of calcareous ores. Sulfuric acid can be microbiologically generated from elemental sulfur (S^0), which would reduce the overall costs and eliminate hazardous sulfuric acid transport. Acidity of the biogenic sulfuric acid was successfully increased by buffering afforded by sulfate/bisulfate couple.

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Table 1. Extremely acidophilic, sulfur-oxidizing prokaryotes used to inoculate the sulfuric acid-generating bioreactor at 30 °C (30SAGB). Microorganisms used to inoculate the sulfuric acid-generating bioreactor at 50 °C (50SAGB) are marked with asterisk.

Prokaryotes	Temperature (T) range	T_{opt}	pH_{opt}	pH_{min}	Metabolism
Acidithiobacillus (At.) albertensis^{T}	10 to 40	25 to 30	3.5 to 4.0	0.5	autotrophic S^0 oxidizer
At. caldus^{T} *	32 to 52	45	2.0 to 2.5	1.0	mixotrophic S^0 oxidizer
Sulfobacillus (Sb.) thermosulfidooxidans^{T}	up to 58	45	2.0	1.1	mixotrophic Fe^{2+}/S^0 oxidizer
Sulfolobus (S.) metallicus^{T} °*	50 to 75	65 to 70	2.0 to 3.0	1.0	autotrophic Fe^{2+}/S^0 oxidizer

° archaeon
* used to inoculate the 50SAGB

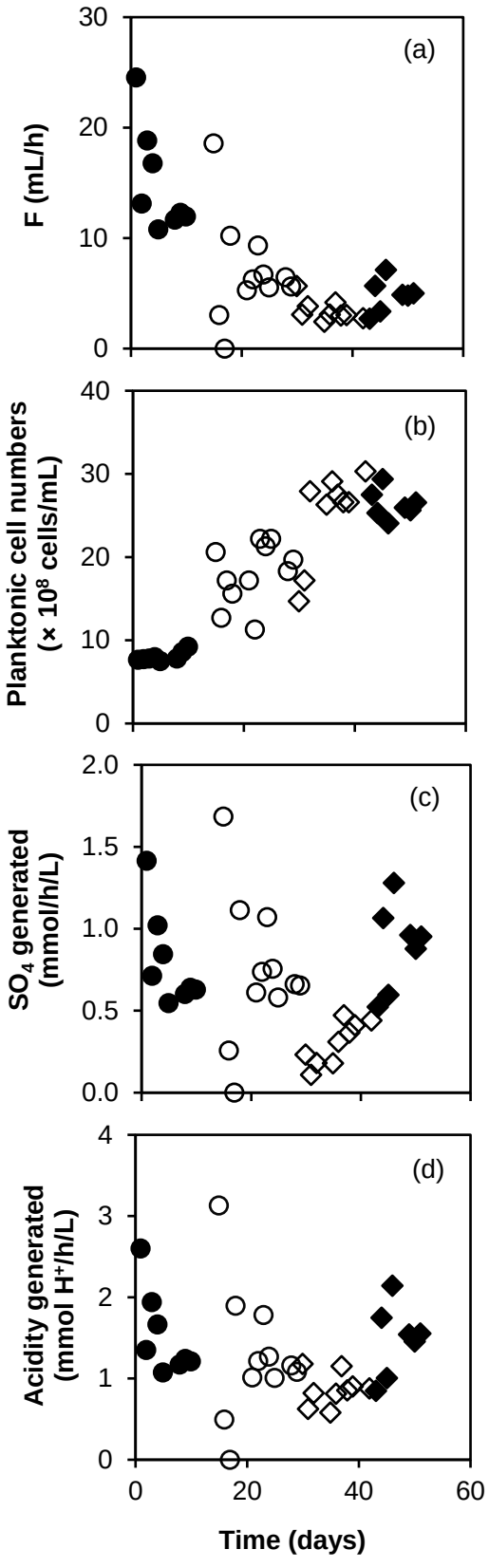

Fig. 1. Changes in (a) flow rates, (b) effluent planktonic cell counts, (c) sulfate and (d) acidity generations (as H^+ concentration) during oxidation of granular elemental sulfur in sulfuric acid-generating bioreactor at 30 °C (30SAGB). Key: (●,■) pH 1.0, and (○,□) pH 0.8 effluents. (●,○) non-amended influent medium, and (■,□) medium amended with 200 mM sulfate.

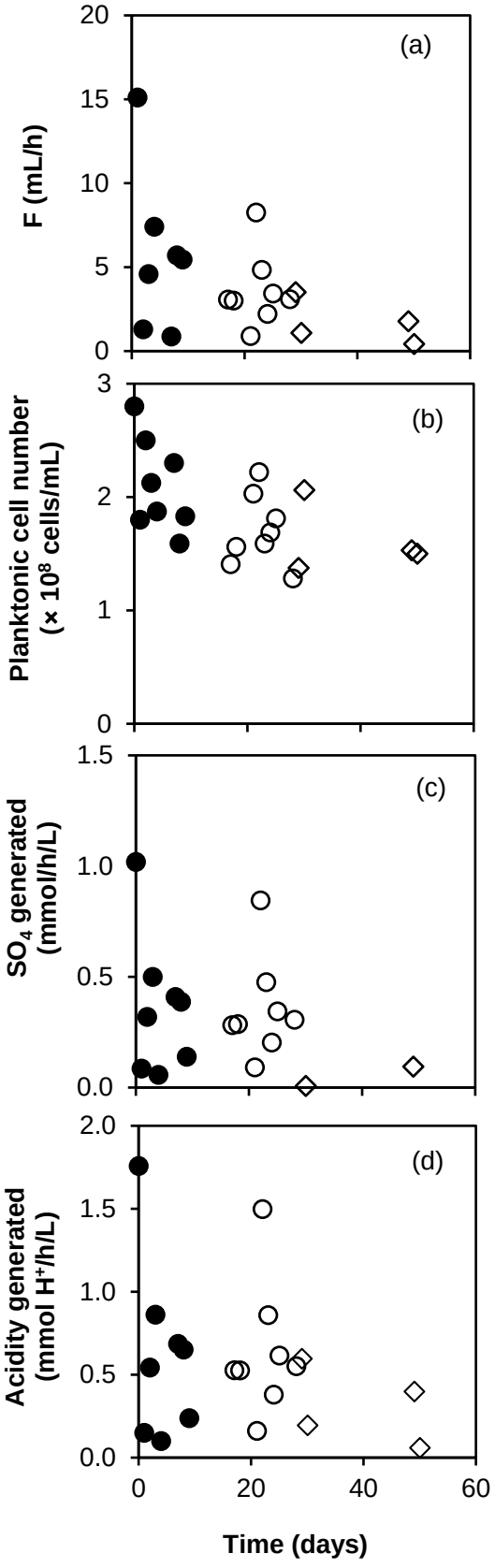

Fig. 2. Changes in (a) flow rates, (b) effluent planktonic cell counts, (c) sulfate and (d) acidity generations (as H^+ concentration) during oxidation of granular elemental sulfur in sulfuric acid-generating bioreactor at 50 °C (50SAGB). Key: (●,■) pH 1.0, and (○,□) pH 0.8 effluents. (●,○) non-amended influent medium, and (■,□) medium amended with 200 mM sulfate.

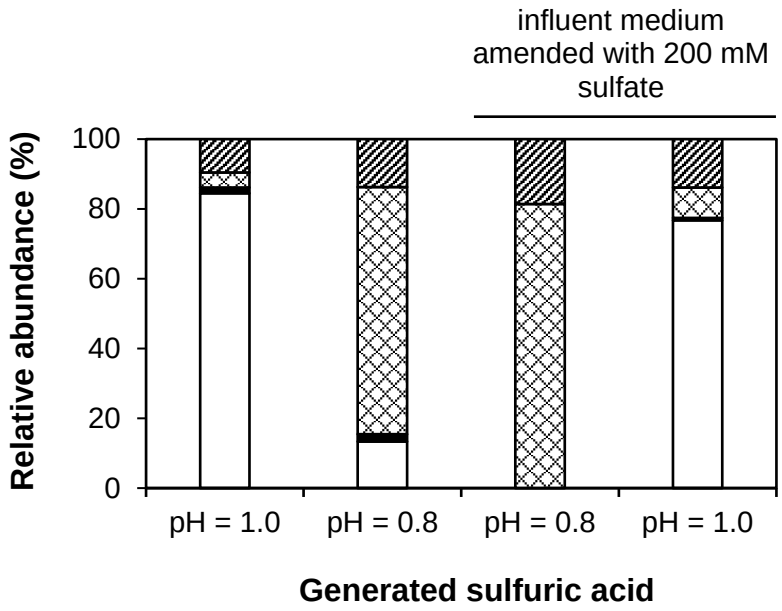

Fig. 3. Relative abundance of acidophilic bacteria in effluent liquors from sulfuric acid-generating bioreactor during oxidation of granular elemental sulfur at 30 °C, as depicted by T-RFLP analysis of amplified 16S rRNA genes digested with HaeIII. Key: (☐) *At. albertensis*, (■) *At. caldus*, (▨) *Sb. thermosulfidooxidans*, (▨) *Acidibacillus* sp.

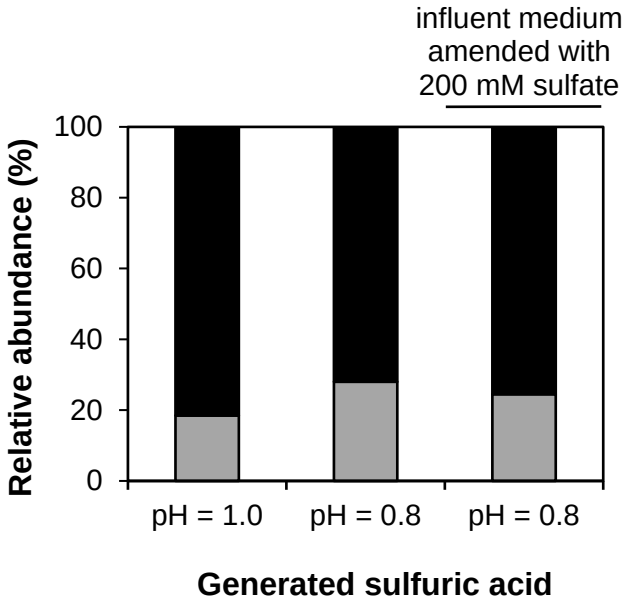

Fig. 4. Relative abundance of acidophilic prokaryotes in effluent liquors from sulfuric acid-generating bioreactor during oxidation of granular elemental sulfur at 50 °C, on the basis of cellular morphologies. Key: (▩) *S. metallicus*, (■) *At. caldus*.