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Biotic factor does not limit operational pH in packed-bed bioreactor for ferrous iron biooxidation

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Abstract Ferrous ion biooxidation is a process with many promising industrial applications: mainly regeneration of ferric ion as an oxidizing reagent in bioleaching processes and depuration of acid mine drainage. The flooded packed-bed bioreactor (FPB) is the design that leads to the highest biooxidation rate. In this bioreactor, biomass is immobilized in a biofilm that consists of an inorganic matrix, formed by precipitated ferric compounds, in the pores of which cells are attached. This biofilm covers the surface of particles (siliceous stone) that form the bed. The chemical stability of this inorganic matrix defines the widest possible pH range in FPBs. At pH below 1, ferric matrix is dissolved and cells are washed out. At pH higher than 2, ferric ion precipitates massively, greatly hindering mass transfer to cells. Thus, among other parameters, pH is recognised as a key factor for operational control in FPBs. This paper aims to explain the effect of pH on FPB operation, with an emphasis on microbial population behaviour. FPBs seeded with mixed inocula were assayed in the pH range from 2.3 to 0.8 and the microbial population was characterised. The microbial consortium in the bioreactor is modified by pH; at pH above 1.3 *Acidithiobacillus ferrooxidans* is the dominant microorganism, whereas at pH below 1.3 *Leptospirillum ferrooxidans* dominates. Inoculum can be adapted to acidity during continuous operation by progressively decreasing the pH of the inlet solution. Thus, in the pH range from 2.3 to 1, the biotic factor does not compromise the bioreactor performance.

Keywords Packed-bed bioreactors · Biofilms · Immobilised cells · Mesophiles · Ferrous ion biooxidation · Mixed inocula

Abbreviations

FPB	Flooded packed-bed bioreactor
IPTG	Isopropyl β-D-1-thiogalactopyranoside
X-GAL	5-Bromo-4-chloro-indolyl-β-D-galactopyranoside

Introduction

The applications of ferrous ion biooxidation $2Fe^{2+} + 2H^{+} + 1/2O_2 \rightarrow 2Fe^{3+} + H_2O$ have been extensively described in the literature. Among those applications are the regeneration of ferric ion as oxidizing reagent and the removal of ferrous ion in the processes of acid mine drainage depuration [20, 27]. For these applications, the bioreactor design leading to the highest oxidation rate (4 kg of ferric per hour and per square metre of base area) is the flooded packed-bed bioreactor (FPB) [15]. This bioreactor has been successfully tested at pilot plant scale, integrated in both hydrometallurgical and environmental processes [1, 6]. The FPB (Fig. 1) consists of a column packed with inert siliceous stone particles constituting the fixed bed. Air and liquor are continuously fed at the bottom, moving upwards through the bed and occupying all the voids. In the bed, each particle is coated by biofilm,

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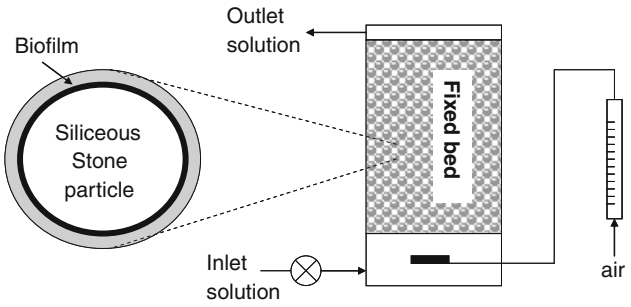

Fig. 1 Scheme of flooded packed-bed bioreactor. The *magnified section* represents a particle from the fixed bed

consisting of an inorganic matrix of precipitated ferric compounds (mainly oxyhydroxides and jarosites) in the pores of which microbial cells are adsorbed [10]. The most common mesophilic microorganisms used in biooxidation applications are *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans.*

Flow in this kind of tubular bioreactor is closer to the model of a stirred tank bioreactor than to that of a plug flow bioreactor model [19]. However, considerable deviations for heights higher than 30 cm have been described; therefore, pH gradients inside the bioreactor must be assumed.

Oxygen transfer limitation in the FPB has been described [19] and relates to the composition of the medium. Ionic strength negatively affects oxygen solubility by means of the salting out effect [17].

The chemical stability of the inorganic matrix greatly determines cell retention. pH has been mentioned as a key operational parameter [12, 13, 23, 32], because it affects the biofilm stability in two ways. On the one hand, pH alters the precipitation equilibria of ferric compounds. From a thermodynamic point of view, the pH in the FPB must be limited (1) at high values to avoid massive precipitation of iron compounds and (2) at low values to avoid dissolution of the inorganic matrix. An excessive accumulation of precipitates over the surface of particles can lead to the clogging of the channels meant for fluids, and to the formation of physical barriers, hindering the diffusion of nutrients and products to the cells. An excessive dissolution of precipitates can lead to cell wash-out. At 31 °C, the optimal temperature for growing the aforementioned mesophilic microorganisms, and pH above 2 ferric ion has a low solubility [4, 8, 14, 30]. Therefore, pH 2 is accepted as the upper limit for operational pH [16]. On the other hand, pH influences the ability of microorganisms to oxidize ferrous ion. For instance, it is well known that *L. ferrooxidans* tolerates more acidic environments than *A. ferrooxidans*. It has been described that *A. ferrooxidans* typically grows at a pH higher than 1.5 and that the optimum pH is between 1.8 and 2.5 [2, 9, 11, 21, 30, 31]. *L. ferrooxidans* typically grows at a pH higher than 1 and its optimum pH is between 1.3 and 2 [2, 3, 9, 21].

For biooxidation applications the chosen inoculum should fulfil a few pH requirements. First of all, they should be versatile i.e. the biological activity of the inoculum should not be adversely affected by medium's pH in the whole range imposed by the chemical stability of the inorganic matrix. Secondly, the chosen inoculum should be flexible, adapting its metabolic activity in a simple and rapid way when facing a pH change. In the industrial domain, pH changes are caused by the operational needs/contingencies, or by the limitations of accuracy and response time intrinsic to the control systems. Certainly, a mixed inoculum fulfils all the aforementioned requirements [24].

We have reported the influence of pH on an FPB [16] and observed that a stable operation is possible in the pH range from 2.3 to 1. For this paper, the objective was to ascertain the role of the biotic factor in setting up this pH range. To this end, we have undertaken the identification of the microbial population in the FPB when the pH is between 2.3 and 0.8. We have observed that pH influences the dominance of microorganisms inoculated into the FPB; pH 1.3 constitutes a transition point for microbial dominance: *acidithiobacilli* dominated over *leptospirilli* above pH 1.3, and *leptospirilli* dominated over *acidithiobacilli* below pH 1.3. Since microbial population behaves dynamically in response to pH changes, the biotic factor does not limit the operational pH range.

Materials and methods

Inocula

Two different inocula, named A and L, were used for seeding. These inocula were obtained from mine acid drainage waters of Rio Tinto Mine, which is located at the Iberian pyritic belt (specifically at Filón Norte, Cerro Colorado, SW Iberian Peninsula). At the time of collection the pH was measured to be around to 2. Inoculum A was maintained by seeding in Silverman and Lundgren's 9K nutrient medium [29]. Inoculum L was maintained on 9K nutrient medium, adjusted to pH 1.25 with sulphuric acid. Both inocula were sub-cultured every week and grown at 31 °C.

Batch biooxidation assays

Three series of consecutive batch culture assays were carried out at pH 1.1, 1.3, 1.5, 1.7 and 1.9, using cultures A and L as inoculum. These assays were conducted in 250-ml Erlenmeyer flasks containing 80 ml of 9K nutrient medium at the selected pH, adjusted with sulphuric acid. The

medium was seeded with 20 ml of inoculum taken from the last culture grown in the same series. The flasks were stirred at 180 rpm in an orbital shaker and kept at 31 °C. The time required for complete oxidation of ferrous ion was measured.

Continuous biooxidation assays

Continuous biooxidation assays were carried out in FPBs (Fig. 1), which consisted of columns (4.2 cm in diameter and 10 cm in height) filled with siliceous stone particles (particle size 6–8 mm). These bioreactors were inoculated with cultures A or L and operated according to Mazuelos et al. [18]. All the tests were performed at 31 °C in 9K nutrient medium ($[Fe^{2+}] = 8.9$ g/l), whose pH was modified with concentrated sulphuric acid to the value set for the operation. The air flow rate was 250 or 500 ml/min, depending on the case. In all tests the liquid flow rate was 100 ml/h in order to avoid the ferrous ion becoming a limiting reagent and the residence times being too short.

As a rule, a steady state was considered when ferrous ion concentration (at the outlet) varied less than 5 % in a period of time equal to 50 times the mean residence time (1 h). For a bed height of 10 cm, the flow pattern in this kind of bioreactor is close to an ideal continuous stirred tank reactor model [19]. Therefore, the pH inside the bioreactor is considered the same as the outlet pH.

Productivity of ferric ion was calculated by the following formula:

$$([Fe^{2+}]_{inlet} - [Fe^{2+}]_{outlet}) \cdot \text{liquid flow rate}$$

Ferrous ion concentration and pH

Ferrous ion concentration was determined by titration with a standard potassium dichromate solution (0.05 N $K_2Cr_2O_7$) and measured in an automatic titrator (Radiometer Copenhagen). The pH was measured with a Sartorius PT-10 pH meter and a WTW pH electrode, calibrated with pH buffers between 1 and 3.

DNA extraction

Samples of 5 ml of each culture medium were used DNA extraction. Cells were sedimented by centrifugation in a tabletop microfuge at maximum speed for 3 min, and the DNA was extracted by using an AquaPure Genomic DNA Isolation Kit, according to supplier's instructions (BioRad). Briefly, cells were disrupted by incubation in lysis buffer for 15 min at 80 °C and 30 min at room temperature. Contaminant RNA and protein were removed by adding RNAse and a precipitation solution, respectively. The total DNA was obtained by means of an isopropanol precipitation and by resuspension in a hydration buffer.

PCR, cloning and sequencing

Amplifications were carried out in 50-µl-volume reactions containing template DNA, 25 µM of each forward and reverse primers (F: 5′–AGAGTTTGATCCTGGCTCAG–3′; R: 5′–TGCGGCTGGATCACCTCCTTT–3′, according to [33]), 200 µM dNTP, 2 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase (Biotools). Cycling involved an initial incubation for 3 min at 94 °C, and then 35 cycles of 30 s at 94 °C (denaturing), 30 s at 51 °C (annealing) and 120 s at 72 °C (extension), followed by a final extension step of 15 min at 72 °C. PCR products were purified using the High Pure PCR Product Purification Kit, following supplier's instructions (Roche). Ligation reactions were performed into pGEM-T Easy Vector, following the supplier's instructions (Promega). Two-microlitre ligation reactions were used to transform *Escherichia coli* XL10-Gold cells by the heat-shock method, following the supplier's instructions (Stratagene). Clones were selected by white/blue screening in LB agar medium containing an appropriate amount of tetracycline, ampicillin, X-GAL and IPTG [26]. Plasmid DNA was isolated from liquid-cultured cells by using the PureYield Plasmid Miniprep System, following the supplier's instructions (Promega). Analysis of plasmid DNA was done by restriction using *Eco*RI (Sigma), agarose electrophoresis and sequencing using an automated method (Sistemas Genómicos, Valencia, Spain). For further analyses, 25 positive clones were selected from each sample.

Sequence analysis

Sequence identity was primary searched by pairwise sequence alignment using the Blast server at The European Ribosomal Database (http://bioinformatics.psb.ugent.be/webtools/rRNA). Primary identification assignment was based on the Blast score, E-value and percentage of identity. Final identity assignment was done based on each particular relationship in a phylogenetic tree. In order to infer phylogenies, we built a data set with those sequences obtained by us and with those retrieved from a public database (www.ncbi.nlm.nih.gov). This latter data set included reference sequences such as those of *Acidithiobacillus*, *Leptospirillum*, *Acidiphilium* and *Ferromicrobium*. The whole sequence data set was analysed using the Robust Phylogenetic Analysis For The Non-Specialist [5]. Briefly, sequences were aligned using MUSCLE, curated using Gblocks, and trees inferred using Bayesian analyses with default settings (www.phylogeny.fr) [5].

Results and discussion

Characterization of inocula A and L

Previously, the population structures of inocula A and L were determined by means of classical isolation/identification microbiological methods. Microorganisms isolated from these cultures were identified as belonging to *A. ferrooxidans*, *L. ferrooxidans* and associated heterotrophs [28]. For this paper, we have characterised the microorganisms in these cultures by DNA extraction, PCR amplification of the small-subunit of rDNA, sequencing and phylogenetic analysis. In this way and for each culture, we obtained a set of 16S rDNA sequences that, along with sequences retrieved from the database, were used to build a whole data set tree (not shown). Sequences in this tree belong to four main groups: sequences like those of *Acidithiobacillus* (*acidithiobacillus* phylotypes), *Leptospirillum* (*leptospirillum* phylotypes) and *Ferrimicrobium* (*ferrimicrobium* phylotypes). Figure 2 is a trimmed version of the aforementioned tree showing *A. ferrooxidans*, *L. ferrooxidans*, *Leptospirillum ferriphilum*, *Leptospirillum ferrodiazotrophum*, *Leptospirillum rubarum*, *Ferrimicrobium* and *Acidimicrobium ferrooxidans*, all obtained from the database, and five sequences representing the whole variability of sequences obtained from cultures A and L (termed respectively Mc9KA and Mc9KL in Fig. 2). The remainder sequences (termed E in Fig. 2) were also obtained by us in the course of the experimentation. The tree in Fig. 2 has the same tree topology as that built with the whole data set.

As shown in Fig. 2, the microorganisms present in inocula A and L are different: only *acidithiobacillus* phylotypes were identified in inoculum A, whereas both *leptospirillum* and *ferrimicrobium* phylotypes were identified in inoculum L. This is in agreement with what has been observed in nature. In the ecosystem of Rio Tinto, from where our inocula were obtained, the more acidic niches are dominated by *leptospirilli*, whereas the less acidic ones are dominated by *acidithiobacilli* [7].

Batch biooxidation assays

In order to test the growth of inocula A and L at different pHs, three series of consecutive batch cultures (R1–R3) were carried out at different pHs. Table 1 shows the results obtained in these series of batch biooxidation assays.

Interestingly, inocula A and L both grew at all tested pHs, though the time required for complete oxidation of ferrous ion varied depending on which inoculum was used for seeding. In the range studied, pH did not exert any effect when inoculum L was used (Table 1). Times required for complete oxidation were similar and the population structure barely changed: only *leptospirillum* phylotypes were identified in these cultures. In contrast, the pH did have an effect when inoculum A was used. When the pH decreases, the time required for complete oxidation increases, and the population structure shifts from *acidithiobacillum* to *leptospirillum* phylotypes. Noticeably, the population transition point was found at pH 1.3. Moreover, the time required for complete oxidation decreased in each subculturing for each series. This latter fact indicates that the microbial population in inoculum A adapts to the medium's acidity.

Continuous biooxidation assays at constant pH

Continuous biooxidation assays were conducted at constant pH in four bioreactors, which differ on the inoculum used and on feed pH during biofilm formation and continuous operation. These bioreactors are termed B1, B2, B3 and B4 (Table 2).

Bioreactors operated at pH 1.25 had different behaviours. B1, seeded with inoculum A, did not form the biofilm (indicated as "–" in Table 2); after three attempts cells were not attached to the support. The fact that inoculum A hardly grows in batch assays at pH lower than 1.3 relates to the inability of the cells to become established in B1 (Table 2). On the contrary, inoculum L was established in B3. It is then assumed that the process for biofilm formation at pH 1.25 would require an inoculum previously adapted to this pH. Productivity in B3 increased when air flow rate increased, indicating oxygen transfer limitation.

Bioreactors B2, at air flow rates of 250 and 500 ml/min, and B4, at an air flow rate 500 ml/min, did not reach a steady state, because outlet the pH is higher than 2.3. It is noteworthy that the microbial population dominating in each case is different. As mentioned in the "Introduction" section, at these pHs the massive precipitation of ferric ion hinders diffusion and flow; therefore, we conclude that bioreactor instability does not depend on the dominant microbial population, but on the equilibrium of ferric ion precipitation.

The highest productivities are obtained with inoculum A, despite the fact that it is more restrictive with respect to the pH than inoculum L. Therefore, once this restriction is overcome, seeding the bioreactor with inoculum A could be more advantageous than seeding it with inoculum L. We tested this idea by seeding a bioreactor with inoculum A at a high pH and then decreasing the pH in a stepwise manner.

Continuous biooxidation assays at variable pH

A bioreactor was operated in the same manner as bioreactor B2, except that the air flow rate was maintained at 500 ml/min and the inlet pH was decreased gradually from

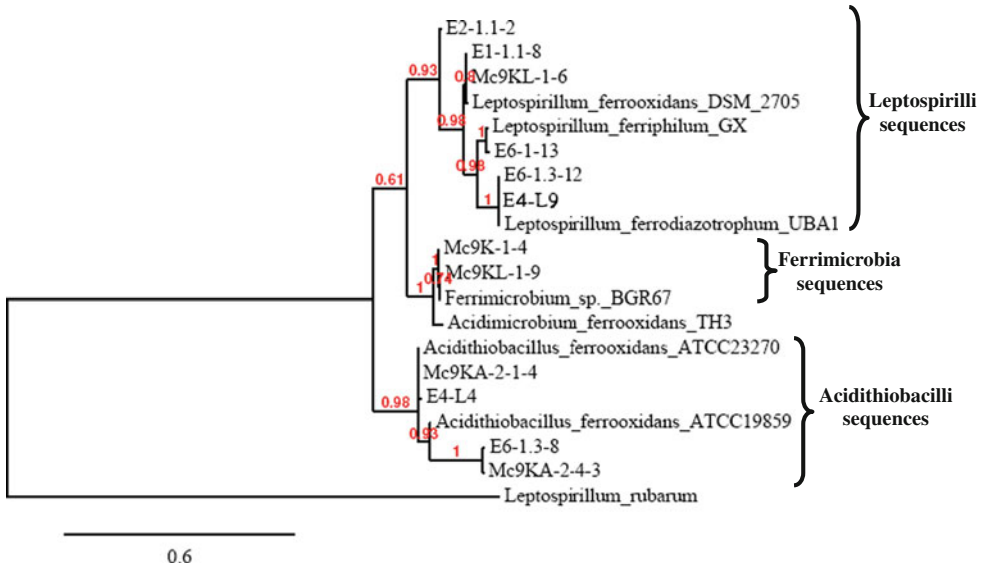

Fig. 2 Bayesian phylogenetic tree of iron oxidisers and representative isolates. This is a trimmed version of phylogenetic tree obtained in the course of the experimentation. The topology of the whole and the trimmed tree is essentially the same. *Numbers* at nodes represent Bayesian posterior probabilities. *Bar scale* represents the number of sequence changes per branch length. GenBank accession numbers are *A. ferrooxidans* TH3 (EF621760.1), *A. ferrooxidans* ATCC 19859 (AF362022.1), *A. ferrooxidans* ATCC 23270 (CP001219.1), *Ferrimicrobium* sp. BGR 67 (GU183398.1), *L. ferriphilum* GX (DQ451017.1), *L. ferrodiazotrophum* (EF065178.1), *L. ferrooxidans* (X86776.1), *L. rubarum* (DS180875.1), E2-1.1-2 (HM769763), E1-1.1-8 (HM769764), Mc9KL-1-6 (HM769765), E6-1-13 (HM769773), E6-1.3-12 (HM769766), E4-L9 (HM769769), Mc9KL-1-9 (HM769768), Mc9KL-1-4 (HM769774), Mc9KA-2-1-4 (HM769769), E4-L4 (HM769770), E6-1.3-8 (HM769771) and Mc9KA-2-4-3 (HM769772) (color figure online)

Table 1 Batch biooxidation assays

Parameter	Batch	Culture A[a] (dominant phylotype *acidithiobacilli*)					Culture L[a] (dominant phylotype *leptospirilli*)				
Initial pH		1.1	1.3	1.5	1.7	1.9	1.1	1.3	1.5	1.7	1.9
Time for complete oxidation (days)	R1	12	5	3	3	3	1	1	1	1	1
	R2	5	2	2	1	1	1	1	1	1	1
	R3	3	2	2	1	1	1	1	1	1	1
Dominant phylotype	R3	Lep	Lep/Ac	Ac	Ac	Ac	Lep	Lep	Lep	Lep	Lep

[a] Inoculum, 20 ml of A or L culture; reactor, 250-ml Erlenmeyer flasks; medium, 80 ml of 9K nutrient medium at the selected pH, adjusted with sulphuric acid; agitation rate, 180 rpm; temperature, 31 °C

Lep and Ac indicate *leptospirilli* and *acidithiobacilli*, respectively

Table 2 Continuous biooxidation assays at constant pH using culture A or L as inoculum

Parameter	Culture A (dominant phylotype *acidithiobacilli*)				Culture L (dominant phylotype *leptospirilli*)			
Bioreactor	B1		B2		B3		B4	
Inlet pH	1.25		1.90		1.25		1.90	
Air flow rate (ml/min)	250	500	250	500	250	500	250	500
Outlet pH	–	–	2.31	2.32	1.36	1.42	2.19	2.32
Maximum productivity (g/h)	–	–	0.44[a]	0.59[a]	0.33	0.43	0.37	0.43[a]
Dominant phylotype	–	–	*Acidithiobacilli*		*Leptospirilli*		*Leptospirilli*	

Reactor, flooded packed-bed bioreactor (4.2 cm in diameter and 10 cm in height); temperature, 31 °C; medium, 9K nutrient medium whose pH is modified with concentrated sulphuric acid to the value set for the operation; air flow rate, 250 or 500 ml/min; liquid flow rate, 100 ml/h

[a] No steady state

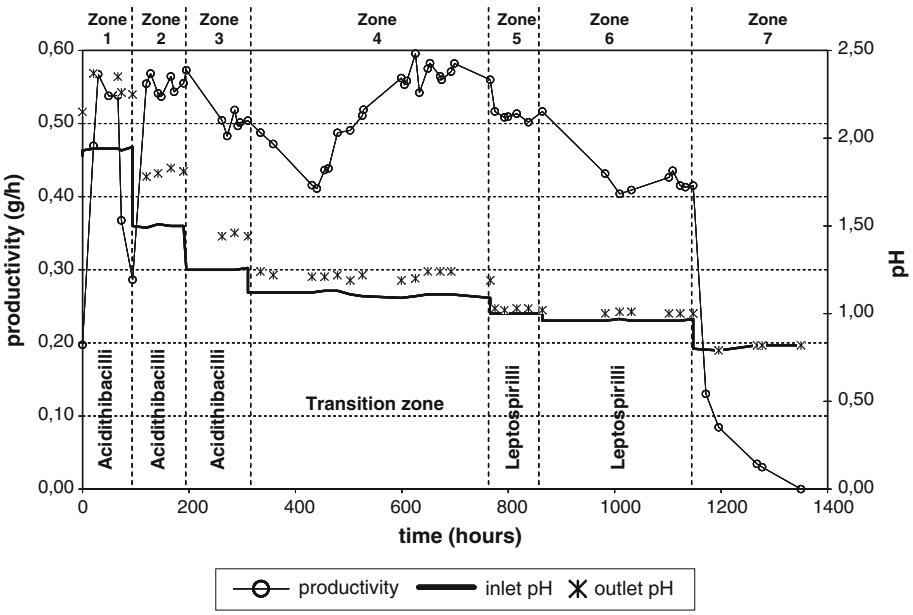

Fig. 3 Continuous biooxidation assays at variable pH. Inoculum, culture A; reactor, flooded packed-bed bioreactor (4.2 cm in diameter and 10 cm in height); temperature, 31 °C; medium, 9K nutrient medium whose pH is modified with concentrated sulphuric acid to the value set for the operation; air flow rate, 500 ml/min; liquid flow rate, 100 ml/h

1.9 to 0.8. The results are shown in Fig. 3, which has been divided in seven zones, each corresponding to a change in feed pH.

From zone 1 to 3, *acidithiobacillum* phylotypes dominate the microbial population in the bioreactor, whereas from zone 4 to 7 *leptospirillum* phylotypes dominate. Zone 4 is therefore the microbial transition zone.

In zone 1, a steady state is not reached, as occurred in bioreactor B2 (see preceding paragraph and Table 2). The productivity suddenly decreases when the pH exceeds 2.3, although this effect is reversible once the outlet pH falls below this value. As stated before, this behaviour is probably due to changes in ferric ion precipitation.

In zone 2, a steady state is reached, because zone 1 instability is amended by decreasing the inlet pH to 1.5. The productivity is as high as that of zone 1 but with the advantage of a stable operation owing to the output pH being around 1.8, a value well outside the range of massive ferric ion precipitation.

In zones 3, 5 and 6, the productivity decreases once the pH is diminished, and a steady state is quickly reached. We assume that the productivity fall is explained by the oxygen solubility decrease due to the salting out effect, and/or dissolution of ferric ion precipitates. Both facts could promote the onset of cell wash-out. Implicitly we can presume oxygen limitation, as occurred in bioreactors B2, B3 and B4 (Table 2).

In zone 4, the productivity also decreases at the beginning but then it increases. A steady state is reached at the end with the highest observed productivities. The outlet pH is around 1.25. In addition, *leptospirillum* phylotypes dominate; thus, a population change has occurred. It is worthy to note, first of all, that productivity in zone 4 in the steady state has increased despite the decrease of oxygen solubility as a result of pH change. Since the flow pattern has not changed, oxygen transfer towards the biofilm should improve. A plausible explanation for this assertion is that the higher acidity has fostered partial dissolution of the ferric ion compounds in the matrix, eliminating diffusional barriers in the biofilm. Secondly, at pH 1.25 *leptospirilli* are not growth-constrained (Table 1) [22]. At optimal growing conditions cells might act as a proton sink that leads to local pH gradients in their vicinity, locally protecting the ferric precipitates matrix from acid attack. Therefore, *leptospirilli* presumably grow attached to the matrix, as they protect it, whereas *acidithiobacilli* are presumably washed out. This is one conceivable reason to explain the observed change in the microbial population. In summary, we hypothesise that a new and more productive biofilm is now present in the bioreactor.

In zone 7, the productivity drops to zero despite that *leptospirilli* are likely to be metabolically active at pH 0.7, as demonstrated by the fact that cells from the outlet stream grew in a 9K medium at pH 0.8 (results not shown). The failure of the bioreactor is due to a complete dissolution of the inorganic matrix; bed particles found in the bioreactor had an appearance similar to that before use (results not shown) i.e. ferric ion precipitates have been completely dissolved, allowing cells to wash out.

Rawling et al. [25] stated that ferric to ferrous ion ratio (redox potential) is the main factor affecting the dominance of one microorganism over another in the biooxidation process. However, they do not exclude that pH also plays an important role. We have observed that pH is responsible for population change because in the FBP ferric to ferrous ion ratios have shown no tendency.

Conclusion

Here, we have reported the effect of pH on the behaviour of the microbial population during flooded packed-bed bioreactor operation. In conclusion, pH constitutes an important variable in FPB operation. In the pH range from 2.3 to 1 the biotic factor does not compromise bioreactor performance; these limits are linked to the chemistry of ferric ion precipitation and are the widest possible operational pH range. However, pH plays an important role in the dominance of microorganisms. When decreasing the pH from 2.3 to 1, the biological population shows dynamic behaviour, although the productivity varies little. pH 1.3 constitutes a transition point: *acidithiobacilli* dominated over *leptospirilli* above pH 1.3, and vice versa. Thus, it is possible to adapt the mixed inoculum to acidity by means of decreasing the pH stepwise inside the bioreactor. We therefore assert that this microbial consortium meets the following technical requirements, with respect to pH, to be used as inoculum in a FPB: versatility and flexibility.

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