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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 drainages. 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 FPB. 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 operation control in FPB.

This paper aims to explain the effect of pH on FPB operation, emphasizing on microbial population behaviour. FPBs seeded with mixed inocula have been assayed in the pH range from 2.3 to 0.8 and microbial population has been characterised. Microbial consortium in bioreactor is modified by pH.; at pH above 1.3 *Acidithiobacillus ferrooxidans* is the dominant microorganism, while at pH below *Leptospirillum ferrooxidans* dominates. Inoculum can be adapted to acidity during continuous operation, progressively decreasing the pH of the inlet solution. Thus, in the pH range from 2.3 to 1, 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

58 **Introduction**

59

60 The applications of ferrous ion biooxidation $2Fe^{2+} + 2H^{+} + \frac{1}{2}O_2 \rightarrow 2Fe^{3+} + H_2O$ have been
61 extensively described in the literature. Among those applications, the regeneration of ferric ion as
62 oxidizing reagent and the removal of ferrous ion in the processes of acid mine drainage depuration [20,
63 27].
64 For these applications, the bioreactor design leading to the highest oxidation rate (4 Kg of ferric per hour
65 and per m^2 of base area) is the flooded packed-bed bioreactor (FPB) [15]. This bioreactor has been
66 successfully tested at pilot plant scale, integrated in both hydrometallurgical and environmental processes
67 [6, 1]. The FPB (Fig. 1) consists of a column packed with inert siliceous stone particles constituting the
68 fixed bed. Air and liquor are continuously fed at the bottom, moving upwards through the bed and
69 occupying all the voids. In the bed, each particle is wrapped around by a biofilm, consisting of an
70 inorganic matrix of precipitated ferric compounds (mainly oxyhydroxides and jarosites) in the pores of
71 which, microbial cells are adsorbed [10]. The most common mesophilic microorganisms used in
72 biooxidation applications are *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans.*
73 Flow in this kind of tubular bioreactor is closer to the model of a stirred tank bioreactor than to that of a
74 plug flow bioreactor model [19]. However, considerable deviations for heights higher than 30 cm have
75 been described; therefore, pH gradients inside the bioreactor must be assumed.
76 Oxygen transfer limitation in the FPB has been described [19] and relates to the composition of the
77 medium. Ionic strength negatively affects oxygen solubility by means of the salting out effect [17].
78 The chemical stability of the inorganic matrix greatly determines cell retention. pH has been mentioned as
79 a key operational parameter [12, 13, 23, 32], since it affects the biofilm stability in two ways.
80 On one hand, pH alters the precipitation equilibria of ferric compounds. From a thermodynamic point of
81 view, pH in FPB must be limited i) at high values to avoid massive precipitation of iron compounds and
82 ii) at low values to avoid dissolution of the inorganic matrix. An excessive accumulation of precipitates
83 over the surface of particles can lead to the clogging of the channels meant for fluids, and to the formation
84 of physical barriers, hindering the diffusion of nutrients and products to the cells. An excessive
85 dissolution of precipitates can lead to cell wash-out. At 31°C, the optimal temperature for growing the
86 aforementioned mesophilic microorganisms, and pH > 2 ferric ion has a low solubility [4, 8, 14, 30].
87 Therefore, pH 2 is accepted as the upper limit for operational pH [16].

88 On the other hand, pH influences the ability of microorganisms to oxidize ferrous ion. For instance, it is
89 well known that *Leptospirillum ferrooxidans* tolerates more acidic environments than *Acidithiobacillus*
90 *ferrooxidans*. It has been described that *Acidithiobacillus ferrooxidans* typically grows at a pH higher
91 than 1.5 and that the optimum pH is between 1.8 and 2.5 [2, 9, 11, 21, 30, 31]. *Leptospirillum*
92 *ferrooxidans* typically grows at a pH higher than 1 and its optimum pH is between 1.3 to 2 [2, 3, 9, 21].
93 For biooxidation applications the chosen inoculum should fulfil a few pH requirements. First of all, they
94 should be versatile i.e., its biological activity should not be adversely affected by medium pH in the
95 whole range imposed by the chemical stability of the inorganic matrix. Secondly, the chosen inoculum
96 should be flexible, adapting its metabolic activity in a simple and rapid way when facing a pH change. In
97 the industrial domain, pH changes are caused by the operation needs/contingencies, or by the limitations
98 of accuracy and response time intrinsic to the control systems. Certainly, a mixed inoculum fulfils all the
99 aforementioned requirements [24].
100 We have reported the influence of pH on a FPB [16] and observed that a stable operation is possible in
101 the pH range from 2.3 to 1. For this paper, the objective was to ascertain the role of the biotic factor in
102 setting up this pH range. To this end, we have undertaken the identification of the microbial population in
103 the FPB when the pH is between 2.3 and 0.8. We have observed that pH influences the dominance of
104 microorganisms inoculated into FPB; pH 1.3 constitutes a transition point for microbial dominance:
105 *acidithiobacilli* dominated over *leptospirilli* above pH 1.3, and *leptospirilli* dominated over
106 *acidithiobacilli* below pH 1.3. Since microbial population behaves dynamically in response to pH
107 changes, the biotic factor does not limit the operational pH range.
108
109 **Materials and methods**
110
111 Inocula
112
113 Two different inocula, named A and L, were used for seeding. These inocula were obtained from mine
114 acid drainage waters of Rio Tinto Mine, which is located at the Iberian pyritic belt (specifically at Filón
115 Norte, Cerro Colorado, SW Iberian Peninsula). At the time of collection the pH was measured to be
116 around to 2. The inoculum A has been maintained by seeding in a Silverman and Lundgren’s 9K nutrient

117 medium [29]. The inoculum L has been maintained on 9K nutrient medium, adjusted to pH 1.25 with
118 sulphuric acid. Both inoculum were sub-cultured every week and grown at 31ºC.
119
120 Batch biooxidation assays
121
122 Three series of consecutive batch culture assays were carried out at pH 1.1, 1.3, 1.5, 1.7 and 1.9, using
123 cultures A and L as inoculum. These assays were conducted in 250 mL Erlenmeyer flasks containing 80
124 mL of 9K nutrient medium at the selected pH, adjusted with sulphuric acid. Medium was seeded with 20
125 mL of inoculum taken from the last culture grown in the same series. The flasks were stirred at 180 rpm
126 in an orbital shaker and kept at 31ºC. Time for complete oxidation of ferrous ion was measured.
127
128 Continuous biooxidation assays
129
130 Continuous biooxidation assays were carried out in flooded packed-bed bioreactors (Fig. 1), which
131 consisted of columns (4.2 cm in diameter and 10 cm in height) filled with siliceous stone particles
132 (particle size 6-8 mm). These bioreactors were inoculated with A or L cultures and operated according to
133 Mazuelos *et al.* [18]. All the tests were performed at 31ºC in a 9K nutrient medium ([Fe^{2+}] = 8.9 g/L),
134 whose pH was modified with concentrated sulphuric acid to the value set for the operation. Air flow rate
135 was 250 mL/min or 500 mL/min, depending on the case. In all tests the liquid flow rate was 100 mL/h in
136 order to avoid both ferrous ion becoming a limiting reagent, and residence times being too short.
137 As a rule, a steady-state was considered when ferrous ion concentration (at the outlet) varied less than 5%
138 in a period of time equal to 50 times the mean residence time (1 h). For a bed height of 10 cm, the flow
139 pattern in this kind of bioreactor is close to an ideal continuous stirred tank reactor model [19]. Therefore,
140 pH inside the bioreactor is considered the same as the outlet pH.
141 Productivity of ferric ion was calculated by the following formula:
142 $([Fe^{2+}]_{inlet} - [Fe^{2+}]_{outlet}) \cdot liquid\,flow\,rate$.
143
144 Ferrous ion concentration and pH
145

146 Ferrous ion concentration was determined by titration with a standard potassium dichromate solution
147 (0.05 N $K_2Cr_2O_7$) and measured in an automatic titrator (Radiometer Copenhagen). The pH was measured
148 with a Sartorius PT-10 pH meter and a WTW pH electrode, calibrated with pH buffers between 1 and 3.
149
150 DNA extraction
151
152 Samples of 5 mL of a culture media were used for this purpose. Cells were sedimented by centrifugation
153 in a tabletop microfuge at maximum speed for 3 minutes, and the DNA was extracted by using an
154 AquaPure Genomic DNA isolation Kit, according to supplier's instructions (BioRad). Briefly, cells were
155 disrupted by incubation in lysis buffer during 15 minutes at 80ºC and 30 minutes at room temperature.
156 Contaminant RNA and protein were removed by adding RNAse and by a precipitation solution,
157 respectively. The total DNA was obtained by means of an isopropanol precipitation and by resuspension
158 in a hydration buffer.
159
160 PCR, cloning, and sequencing
161
162 Amplifications were carried out in 50 µL volume reactions containing template DNA, 25 µM of each
163 forward and reverse primers (F: 5'-AGAGTTTGATCCTGGCTCAG-3'; R: 5'-
164 TGCGGCTGGATCACCTCCTTT-3', according to [33]), 200 µM dNTP, 2 mM $MgCl_2$, and 1 U of *Taq*
165 DNA polymerase (Biootols). By cycling we understand: an initial incubation for 3 min at 94°C, and then
166 35 cycles of 30 s at 94°C (denaturing), 30 s at 51°C (annealing), and 120 s at 72°C (extension), followed
167 by a final extension step of 15 min at 72°C. PCR products were purified using the High Pure PCR
168 Product Purification Kit, following supplier's instructions (Roche). Ligation reactions were performed
169 into pGEM-T Easy Vector, following supplier's instructions (Promega). Two µl of ligation reactions were
170 used to transform *Escherichia coli* XL10-Gold cells by the heat-shock method, following supplier's
171 instructions (Stratagene). Clones were selected by white/blue screening in LB agar medium containing an
172 appropriate amount of tetracycline, ampicillin, X-GAL, and IPTG [26]. Plasmid DNA was isolated from
173 liquid-cultured cells by using the PureYield Plasmid Miniprep System, following supplier's instructions
174 (Promega). Analysis of plasmid DNA was done by restriction using *Eco*RI (Sigma), agarose

175 electrophoresis, and sequencing using an automated method (Sistemas Genómicos, Valencia, Spain). For
176 further analyses, 25 positive clones were selected from each sample.
177
178 Sequence analysis
179
180 Sequence identity was primary searched by pair-wise sequence alignment using the Blast server at The
181 European Ribosomal Database (http://bioinformatics.psb.ugent.be/webtools/rRNA). Primary
182 identification assignment was based on the Blast score, E-value and percentage of identity. Final identity
183 assignment was done based on each particular relationship in a phylogenetic tree. In order to infer
184 phylogenies, we built a dataset with those sequences obtained by us and with those retrieved from a
185 public database (www.ncbi.nlm.nih.gov). This latter dataset included reference sequences such as those of
186 *Acidithiobacillus*, *Leptospirillum*, *Acidiphilium*, and *Ferromicrobium*. The whole sequence dataset was
187 analyzed using the Robust Phylogenetic Analysis For The Non-Specialist [5]. Briefly, sequences were
188 aligned using MUSCLE, curated using Gblocks, and trees inferred using Bayesian analyses with default
189 settings (www.phylogeny.fr) [5].
190
191 **Results and discussion**
192
193 Characterization of inocula A and L
194
195 Previously, the population structure of A and L inocula were determined by means of classical
196 isolation/identification microbiological methods. Microorganisms isolated from these cultures were
197 identified as belonging to *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, and associated
198 heterotrophs [28]. For this paper, we have characterised the microorganisms in these cultures by DNA
199 extraction, PCR amplification of the small-subunit of rDNA, sequencing and phylogenetic analysis. In
200 this way and for each culture, we obtained a set of 16 S rDNA sequences that, along with sequences
201 retrieved from the database, were used to build a whole dataset tree (not shown). Sequences in this tree
202 belong to four main groups: sequences like those of *Acidithiobacillus* (*acidithiobacillus phylotypes*),
203 *Leptospirillum* (*leptospirillum phylotypes*), and *Ferrimicrobium* (*ferrimicrobium phylotypes*). Fig. 2 is a
204 trimmed version of the aforementioned tree showing *Acidithiobacillus ferrooxidans*, *Leptospirillum*

205 *ferrooxidans*, *Leptospirillum ferriphilum*, *Leptospirillum ferrodiazotrophum*, *Leptospirillum rubarum*,
206 *Ferrimicrobium*, *Acidimicrobium ferrooxidans*, all obtained from the database, and five sequences
207 representing the whole variability of sequences obtained from cultures A and L (termed respectively
208 Mc9KA and Mc9KL in Fig. 2). The remainder sequences (termed E in Fig. 2) were also obtained by us in
209 the course of the experimentation. The tree in Fig. 2 has the same tree topology than that built with the
210 whole dataset.
211 As seen in Fig. 2, the microorganisms present in A and L inocula are different: only *acidithiobacillus*
212 *phylotypes* were identified in inoculum A, while both *leptospirillum* and *ferrimicrobium phylotypes* were
213 identified in inoculum L. This is in agreement with what has been observed in nature. In the ecosystem of
214 Rio Tinto, from where our inocula were obtained, the more acidic niches are predominated by
215 *leptospirilli*, while the less acidic ones are predominated by *acidithiobacilli* [7].
216
217 Batch biooxidation assays
218
219 In order to test the growth of A and L inocula at different pHs, three series of consecutive batch cultures
220 were carried out at different pHs. Table 1 shows the results obtained in these series of batch biooxidation
221 assays.
222 Interestingly, both A and L inocula grew at all tested pHs, though time for complete oxidation of ferrous
223 ion varied depending on which inoculum was used to seed. In the range studied, pH did not exert any
224 effect when the L inoculum was used (Table 1). Times for complete oxidation were similar and the
225 population structure barely changed: only *leptospirillum phylotypes* were identified in these cultures.
226 Differently, pH did affect when the A inoculum was used. When pH decreases, time for complete
227 oxidation increases, and population structure shifts from *acidithiobacillum* to *leptospirillum phylotypes*.
228 Noticeably, the population transition point was found at pH 1.3. Moreover, time for complete oxidation
229 decreased in each subculturing for each series. This latter fact indicates that the microbial population in A
230 inoculum is adapting to a medium acidity.
231
232 Continuous biooxidation assays at constant pH
233

234 Continuous biooxidation assays were conducted at constant pH in four bioreactors, which differ on the
235 inoculum used and on feed pH during biofilm formation and continuous operation. These bioreactors are
236 termed B1, B2, B3 and B4 (Table 2).
237 Bioreactors operated at pH 1.25 had different behaviours. B1, seeded with A inoculum, did not form the
238 biofilm (indicated as – in Table 2); after three attempts cells were not attached to support. The fact that A
239 inoculum hardly grow in batch assays at pH lower than 1.3 relates to inability of cells to establish in B1
240 (see Table 2). On the contrary, L inoculum was established in B3. It is then assumed that the process for
241 biofilm formation at pH 1.25 would require an inoculum previously adapted to this pH. Productivity in
242 B3 increased when air flow rate increased, indicating oxygen transfer limitation.
243 Bioreactors B2 at air flow rates 250 and 500 mL/min, and B4 at air flow rate 500 mL/min, did not reach a
244 steady-state, because outlet pH is higher than 2.3. Noteworthy, the microbial population dominating in
245 each case is different. As mentioned in the introduction section, at these pHs the massive precipitation of
246 ferric ion entails diffusion and flow hindrances, therefore we conclude that bioreactor instability does not
247 depend on the dominant microbial population, but on equilibrium of precipitation of ferric ion.
248 The highest productivities are obtained with A inoculum, despite the fact that it is more restrictive with
249 respect to the pH than L inoculum. Therefore, once this restriction is overcome, seeding bioreactor with A
250 inoculum could be more advantageous than seeding it with L inoculum. We tested this idea seeding a
251 bioreactor with A inoculum at a high pH and then decreasing the pH in a stepwise manner.
252
253 Continuous biooxidation assays at variable pH
254
255 A bioreactor was operated in the same manner as B2 bioreactor, except that air flow rate was maintained
256 at 500 ml/min and inlet pH was decreased gradually from 1.9 to 0.8. Results are shown in Fig. 3, which
257 has been divided in 7 zones, each corresponding with a change in feed pH.
258 From zone 1 to 3, *acidithiobacillum phylotypes* dominate microbial population in the bioreactor, while
259 from zone 4 to 7 *leptospirillum phylotypes* are dominant. Zone 4 is therefore the microbial transition
260 zone.
261 In zone 1, steady-state is not reached, as occurred in bioreactor B2 (see precedent paragraph and Table 2).
262 Productivity suddenly decreases when pH exceeds 2.3, although this effect is reversible once the outlet

263 pH falls below this value. As stated before, this behaviour is probably due to changes in ferric ion
264 precipitation.
265 In zone 2, steady-state is reached, since zone 1 instability is amended by decreasing the inlet pH to 1.5.
266 Productivity is as high as that of zone 1 but with the advantage of a stable operation due to the output pH
267 being around 1.8, value well outside the range of massive ferric ion precipitation.
268 In zones 3, 5, and 6 productivity decreases once pH is diminished, and steady-state is quickly reached.
269 We assume productivity fall is explained by oxygen solubility decrease due to salting out effect, and/or
270 dissolution of ferric ion precipitates. Both facts could promote the onset of cell wash-out. Implicitly we
271 can presume oxygen limitation, as occurred in B2, B3 and B4 bioreactors (see Table 2).
272 In zone 4, productivity also decreases at the beginning but then it increases. A steady-state is reached at
273 the end with the highest observed productivities. Outlet pH is around 1.25. In addition, *leptospirillum*
274 *phylotypes* dominates; a population change has occurred. It is worthy to note, first of all, that productivity
275 in zone 4 in the steady-state has increased despite the decrease of oxygen solubility as the result of pH
276 change. Since the flow pattern has not changed, oxygen transfer towards the biofilm should improve. A
277 plausible explanation is that the higher acidity has fostered partial dissolution of the ferric ion compounds
278 in the matrix, eliminating diffusional barriers in the biofilm. Secondly, at pH 1.25 *leptospirilli* is not
279 growth-constrained (see Table 1) [22]. At optimal growing conditions cells might act as a proton sink that
280 leads to local pH gradients in their vicinity, locally protecting the ferric precipitates matrix from acid
281 attack. Therefore, *leptospirilli* presumably grows attached to the matrix, as they protect it, while
282 *acidithiobacilli* is presumably washed-out. This is one conceivable reason to explain the observed change
283 in the microbial population. In summury, we hypothesise a new and more productive biofilm is now
284 present in the bioreactor.
285 In zone 7 productivity drops to zero despite that *leptospirilli* are likely to be metabolically active at pH
286 0.7, as demonstrated by the fact that cells from the outlet stream grew in a 9K medium pH 0.8 (results not
287 shown). The failure of the bioreactor is due to a complete dissolution of the inorganic matrix; bed
288 particles found in the bioreactor had an appearance similar to that before use (results not shown) i.e.,
289 ferric ion precipitates have been completely dissolved, allowing cells wash-out.
290 Rawling et al. [25] have stated that ferric to ferrous ion ratio (redox potential) is the main factor affecting
291 dominance of one microorganism over the other in the biooxidation process. However, they do not

292 exclude that pH also plays an important role. We have observed that pH is responsible for population
293 change because in the FBP ferric to ferrous ion ratio have shown no tendency.
294
295 **Conclusions**
296 Here, we have reported the effect of pH on the behaviour of the microbial population during flooded
297 packed-bed bioreactor operation. In conclusion, pH constitutes an important variable in FPB operation. In
298 the pH range from 2.3 to 1 the biotic factor does not compromise bioreactor performance; these limits are
299 linked to chemistry of ferric ion precipitation and are the widest possible operational pH range. However,
300 pH plays an important role in the dominance of microorganisms. When decreasing the pH from 2.3 to 1,
301 the biological population shows dynamic behaviour, although the productivity poorly varies. pH 1.3
302 constitutes a transition point: *acidithiobacilli* dominated over *leptospirilli* above pH 1.3, and vice versa.
303 Thus, it is possible to adapt mixed inoculum to acidity by means of decreasing the pH stepwise inside the
304 bioreactor. We therefore assert that this microbial consortium meets the technical requirements, with
305 respect to pH, to be used as inoculum in a FPB: versatility and flexibility.
306
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308
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311
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Table captions:

Table 1 Batch biooxidation assays. 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: 31ºC. Lep and Ac indicate *leptospirilli* and *acidithiobacilli*, respectively.

Table 2 Continuous biooxidation assays at constant pH. Inoculum: A or L culture. Reactor: flooded packed-bed bioreactor (4.2 cm in diameter and 10 cm in height). Temperature: 31ºC. Medium: 9K nutrient medium whose pH is modified with concentrated sulphuric acid to the value set for the operation. Air flow rate: 250 mL/min or 500 mL/min. Liquid flow rate: 100 mL/h.* indicates no steady-state.

401 **Table 1**

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Inoculum		Culture A					Culture L				
Dominant phylotype		*Acidithibacilli*					*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

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411 **Table 2**

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Inoculum	Culture A				Culture L			
Dominant phylotype	*Acidithiobacilli*				*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*	0.59*	0.33	0.43	0.37	0.43*
Dominant phylotype	-	-	*Acidithiobacilli*		*Leptospirilli*		*Leptospirilli*	

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417 **Figure captions:**

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

421

422 **Fig. 2** Bayesian phylogenetic tree of iron oxidisers and representative isolates. This is a
423 trimmed version of phylogenetic tree obtained in the course of the experimentation. The
424 topology of the whole and the trimmed tree is essentially the same. Red numbers at
425 nodes represent Bayesian posterior probabilities. Bar scale represents the number of
426 sequence changes per branch length GenBank accession numbers are: *Acidimicrobium*
427 *ferrooxidans* TH3 (EF621760.1), *Acidithiobacillus ferrooxidans* ATCC 19859
428 (AF362022.1), *A. ferrooxidans* ATCC 23270 (CP001219.1), *Ferrimicrobium* sp. BGR
429 67 (GU183398.1), *Leptospirillum ferriphilum* GX (DQ451017.1), *L. ferrodiazotrophum*
430 (EF065178.1), *L. ferrooxidans* (X86776.1), *L. rubarum* (DS180875.1), E2-1.1-2
431 (HM769763), E1-1.1-8 (HM769764), Mc9KL-1-6 (HM769765), E6-1-13 (HM769773),
432 E6-1.3-12 (HM769766), E4-L9 (HM769769), Mc9KL-1-9 (HM769768), Mc9KL-1-4
433 (HM769774), Mc9KA-2-1-4 (HM769769), E4-L4 (HM769770), E6-1.3-8
434 (HM769771), Mc9KA-2-4-3 (HM769772).

435

436 **Fig. 3** Continuous biooxidation assays at variable pH. Inoculum: A culture. Reactor:
437 flooded packed-bed bioreactor (4.2 cm in diameter and 10 cm in height). Temperature:
438 31ºC. Medium: 9K nutrient medium whose pH is modified with concentrated sulphuric
439 acid to the value set for the operation. Air flow rate: 500 mL/min. Liquid flow rate: 100
440 mL/h.

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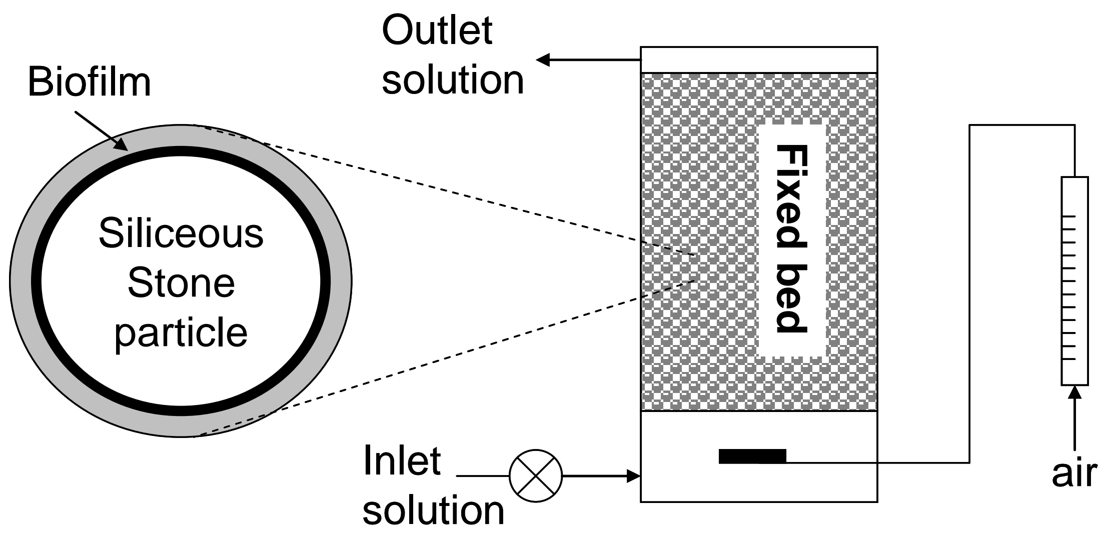
443 **Fig. 1**



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446 **Fig. 2**

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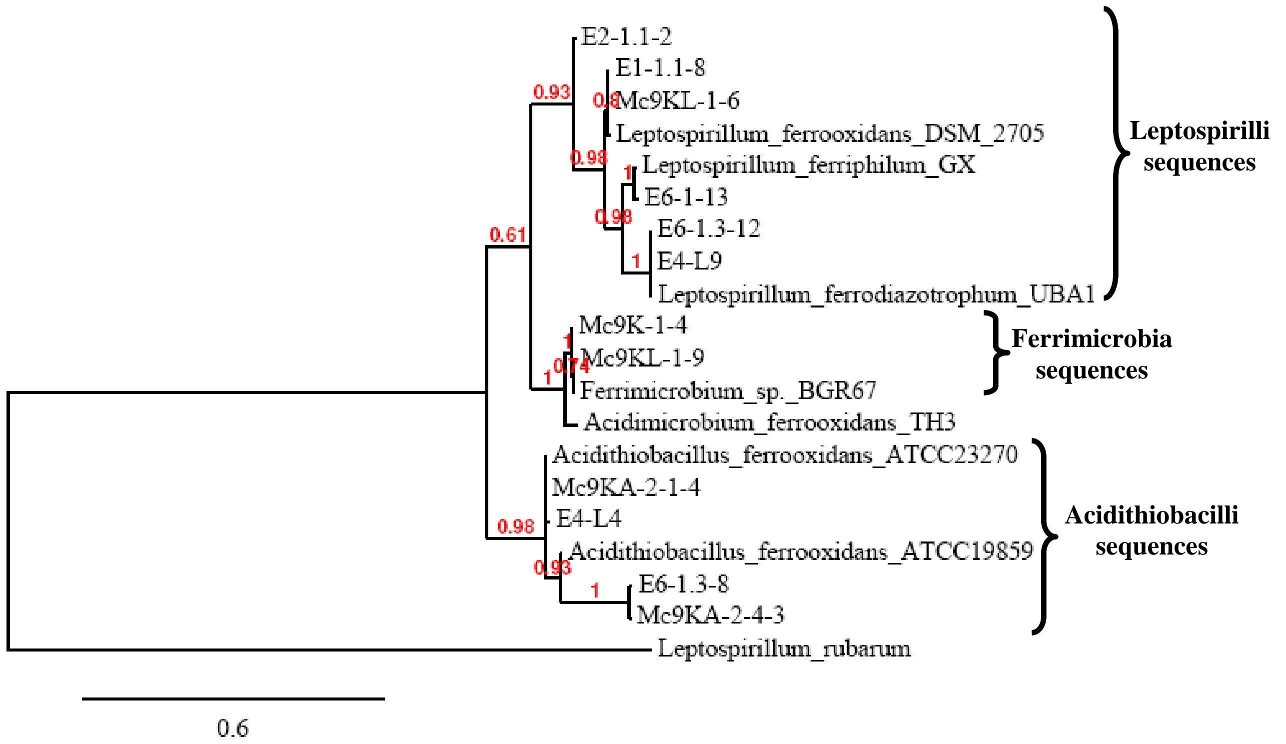

452 **Fig. 3**

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