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Batch kinetics of ferrous iron oxidation by *Leptospirillum ferriphilum* at moderate to high total iron concentration

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ABSTRACT

A study of the kinetics of ferrous iron oxidation by a free suspended culture of the bacterium *Leptospirillum ferriphilum* in batch regime at moderate to high iron concentrations was conducted. A circulating bed airlift bioreactor was used in order to obtain reliable biokinetic data, unaffected by biofilm growth. The two major factors in consideration were the effects of the pH and the total iron concentration in the range of 5–40 g/L. The optimal pH was found between 1.05 and 1.80. In this range a strictly growth associated biooxidation with constant yield coefficient was proven, while at suboptimal pH values non-growth associated iron biooxidation was shown at pH as low as 0.4. This effect was taken into consideration for the derivation of a Monod-type kinetic model, derived on first principles from the electrochemical-enzymatic model for ferrous iron biooxidation. Our model shows a linear dependence between the apparent half-saturation constant (K_{app}) and the total iron concentration in studied range of iron concentration.

1. Introduction

The present work follows more than 50 years of intense research on the iron oxidizing microorganisms and their role in the bioleaching of metals [1,2], and the related processes for the treatment of sour gases and acid mine waters [3,4]. Until recently, this research was mostly focused on the bacterium *Acidithiobacillus ferrooxidans*; however, during the past decade, the members of genus *Leptospirillum* have been increasingly gaining attention as alternative iron oxidizers. One of the main reasons for this shift in interest can be attributed to the fact that although the *Leptospirilli* have a slower growth rate compared to the *Acidithiobacilli*, they tolerate lower pH, higher redox potential of the medium and higher cultivation temperature [5]. These properties arguably make the *Leptospirillum* species a potential candidate for the development of new technologies based on the biological ferrous iron oxidation process. Some potential applications can be seen in the fields of biomachining [6,7], nanoparticle synthesis [8] and microbial fuel cells [9]. The optimization of such processes arguably requires a good understanding of the kinetic behavior of the *Leptospirilli* in a wide variety of conditions, including situations which would not be considered favorable from a purely biological vantage point. Of the two most common species in the genus: *Leptospirillum ferrooxidans* and *Leptospirillum ferriphilum*, the latter is more extremophile and tolerates lower pH and higher cultivation temperature [10]; therefore, it was chosen as the target bacterium of this study.

A brief review of several kinetic studies of *L. ferriphilum* shows how the bacterial growth and biooxidation rates are affected by the pH of the medium, the temperature and the iron concentrations. Previously, the optimal cultivation pH has been found to be in the range of 1.3–1.8 [10], 1.2–2.2 [11] and 0.9–1.5 [12]. Ferrous iron biooxidation and biomass growth at pH below 1 have been reported in continuous operation with an immobilized culture [12]. The same culture was later used for batch experiments at pH below 1, wherein biomass carrying material was withdrawn from the continuously operating biofilm reactor and sonicated to release the immobilized cells [13,14]. In both cases the initial biomass concentration was too high to detect any significant biomass growth; therefore, the growth kinetics was not examined. The temperature effect has been studied in several previous studies [11,14–17], giving an optimal temperature of around 37–42 °C.

In continuous mode of operation, the effects of the iron concentration and speciation for suspended cells of *L. ferriphilum* have been investigated in a wide range of dilution rates, albeit at relatively low iron concentrations of less than 12 g Fe/L [18], and for the closely related *L. ferrooxidans* in a narrow range of dilution rates at 9 and 18 g Fe/L [19]. Both studies are discussed in Section 4.5.

In batch regime the effects of ferrous and ferric iron concentrations have been researched for initial substrate concentrations up to 20 g Fe^{2+}/L with added 0–60 g Fe^{3+}/L [13]. However, in that particular study the high initial biomass concentration precluded the determination of the biomass growth. Additionally it was apparent that the fitness of the chosen kinetic model for ferrous iron biooxi-

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Nomenclature

A, A', B	constants in Eqs. (9)–(15), independent on the substrate concentration
a, c	ionic activity and concentration (g/L)
E_h	oxidation–reduction (redox) potential of the liquid (mV)
E^0, E'	standard and formal redox potentials for Fe^{3+}/Fe^{2+} (mV)
K_2, K_I	kinetic constants in the Meruane model
K_S, K_{app}	half-saturation and apparent half-saturation constants (g Fe/L)
P	product (ferric iron) concentration (g Fe^{3+}/L)
q, q_{max}	specific and maximum specific biooxidation rates (g Fe/(L h))
R^2	coefficient of determination (– or %)
S	substrate (ferrous iron) concentration (g Fe^{2+}/L)
S_{tot}	total iron concentration (g Fe/L)
t	batch cultivation time (h)
t_{lag}	duration of the lag phase (h)
X	cell concentration (cells/L)
Y	biomass yield coefficient (cells/g Fe)

Greek letters

γ	ionic activity coefficient
μ, μ_{max}	specific and maximum specific growth rates (h^{-1})

dation decreased at total iron concentrations above 10 g/L with the correlation coefficient between the model and the experimental data dropping to $R^2 \approx 0.90$.

Based on the above review, we can conclude that while many aspects of the kinetic behavior of the *L. ferriphilum* have been studied, there is a certain lack of knowledge when it comes to the bacterial behavior during the batch cultivation at low pH and high initial ferrous iron concentration. The current article seeks to fill these gaps, as it studies the effects of the pH and the total iron at iron concentrations as high as 40 g/L. Finally, we also aim to develop a kinetic model equation for the iron biooxidation, adequate to such conditions.

2. Materials and methods

2.1. Bacterial culture and growth medium

Acid mine drainage from Iron Mountain Mine, near Redding, California, USA was used as the source for the microbial culture. The mixed culture was incubated in shake flaks using modified 9K synthetic medium [20] with the following composition: 2.0 g/L $(NH_4)_2SO_4$; 0.5 g/L K_2HPO_4; 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 0.1 g/L KCl. The ferrous iron concentration was set with $FeSO_4 \cdot 7H_2O$ to 5 g Fe^{2+}/L, and the pH was set to 1.1 with concentrated H_2SO_4. The same medium with varying Fe^{2+} concentrations and pH was used for the kinetic experiments. All chemicals were of analytical grade.

Following a series of reinoculations in the said medium, the culture was transferred to a continuous bioreactor, where it was maintained at 40 °C and 10 g/L total iron concentration. Before the kinetic experiments were undertaken, it was proven that the culture was highly enriched in *L. ferriphilum* using fluorescence *in situ* hybridization (FISH) and PCR-DGGE genetic analysis (see below). For consequent inoculations the spent medium from the bioreactor was concentrated to approximately 3×10^{12} cells/L by centrifugation at 13,000 × *g* for 20 min and resuspension in an iron-free culture medium, set at pH 1.2. The concentrates were prepared not earlier than 1 day before the kinetic experiments took place and stored at 4 °C prior to being used.

2.2. Genetic analysis of the microbial culture

The bacterial composition of the culture was determined using two independent techniques: fluorescence *in situ* hybridization (FISH), and polymerase chain reaction, followed by denaturing gradient gel electrophoresis (PCR-DGGE). Due to the high total iron concentration in the culture medium, sample preparation for both techniques required initial stripping of the iron. Iron binding and washing was achieved in a two steps process. Firstly, a sample from the culture medium was centrifuged at 13,000 × *g* for 20 min and the cells-free supernatant was decanted. The pellet consisting of microbial cells, and in some cases precipitated iron complexes, was resuspended in 10% oxalic acid. The oxalic acid would dissolve and bind any iron complexes, including jarosite and ferric hydroxides, which are insoluble in water. The second step included centrifugation of the microbial cells from their suspension in the oxalic acid and consequent resuspension of the microbial cell pellet in phosphate buffered saline (PBS; 137 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4 and 2.7 mM KCl).

The slide preparations for FISH were made according to a protocol descried elsewhere [21]. Hybridization was conducted for 90 min at 46 °C with probes specific for the *Leptospirillum* spp. and *Ferroplasma* genus (Table 1). Finally, the samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich) [22]. Slides were observed under visible and UV light with the following equipment: Zeiss Axioskop 40 upright microscope with an HBO 50W UV light source and a 10 bit grayscale camera QICAM (QImaging Corp.), connected to a computer and monitor. Filter sets Chroma C-73347, Zeiss 00 (488000-0000) and Zeiss 50 (488050-0000) were used for viewing the fluorescence from DAPI, Texas Red (with probe LF655) and Cy5 (with probe FER656), respectively.

Further genetic analysis of the enriched culture was performed using the PCR-DGGE technique, followed by a partial sequencing of the 16S rRNA gene and a comparison with available DNA sequences in the NCBI GenBank. The total genomic DNA was extracted with UltraClean™ Soil DNA Isolation Kit, and PCR proceeded with universal primers 341f-GC and 907r [23,24]. The PCR amplification reaction was performed in a Mastercycler thermal cycler (Eppendorf) with the following program: initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 60 s and primer extension at 72 °C for 90 s; and a final extension at 72 °C for 10 min. DGGE was performed on a Decode Universal Mutation Detection System (Bio-Rad). The PCR products were loaded on an 8% (w/v) polyacrylamide gel in 1× TAE with a denaturing gradient spanning from 20 to 70% (100% denaturing gradient corresponds to 7 M urea and 40% (v/v) formamide). The electrophoresis was run at 60 °C and 130 V for 4.5 h. After staining with ethidium bromide, individual DNA-congaing gel fragments were cut and incubated in 50 μL of 1× PCR buffer for 30 min at 4 °C to allow the DNA to leach out into the solution. Subsequent PCR was performed with templates of 1 μL of the eluted DNA, following the same PCR program as above, except for the GC-clamp of the forward primer. The PCR products from the second reaction were purified with the QIAquick PCR purification kit (QIAGEN) and sent for sequencing to the DNA Sequencing Facility at the Robarts Research Institute in London, Ontario, Canada.

2.3. Analytical methods

2.3.1. Cell counting

The bacterial cell concentration was determined through direct manual counting using a transmitted light optical microscope Axioskop 40 (Carl Zeiss, Germany) with 1000× magnification,