Vol. 21, No. 1(2022) IA2575
Revista Mexicana de Ingeniería Química

Characterization of the biological sulfide oxidation process: *in situ* pulse respirometry and *ex situ* pulse microrespirometry approach

Caracterización del proceso biológico de sulfuro oxidación: un enfoque de respirometría de pulsos *in situ* y microrespirometria de pulsos *ex situ*

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Received: August 21, 2021; Accepted: December 25, 2021

Abstract

During the optimization of environmental biotechnology processes, it is important to count on proper and reliable information about the kinetic and stoichiometric parameters of the microorganisms. In this work, the biological sulfide oxidation process was assessed under two novel dynamic pulse respirometric approaches: *in situ* pulse respirometry conducted in airlift bioreactor and *ex situ* pulse respirometry carried out in microreactors (microrespirometry) with samples taken from the airlift bioreactor. The process was characterized in terms of the growth yield, substrate oxidation yield, maximum oxygen uptake rate, affinity constant, and mass transfer coefficient. The *in situ* pulse respirometry showed to be a reproducible technique that allowed the determination of the kinetic and stoichiometric parameters besides the detection of mass transfer limitations in the airlift bioreactor, however its use in biological sulfide oxidation is limited to a few experiments and experimental conditions with significant time investment. On the other hand, the *ex situ* pulse microrespirometry allowed the acquisition of a higher amount of information under a broader range of sulfide concentrations (from 5 to 60 mg H_2S L^{-1}) and experimental conditions such as different pH values. The results obtained showed that the *ex situ* microrespirometry technique would be preferable over *in situ* pulse respirometry for the proper and reliable characterization of the sulfide oxidation process.

Keywords: pulse microrespirometry, sulfide oxidation, airlift bioreactor, microreactor, inhibition models.

Resumen

Durante la optimización de los procesos de biotecnología ambiental es importante contar con información adecuada y confiable sobre los parámetros cinéticos y estequiométricos de los microorganismos. En este trabajo, se evaluó el proceso biológico de oxidación de sulfuro bajo dos nuevos enfoques de respirometría de pulsos: Respirometría de pulsos *in situ* realizada en un bioreactor airlift y respirometría de pulsos *ex situ* realizada en microreactores con muestras del bioreactor airlift (microrespirometría). El proceso se caracterizó en términos del rendimiento de crecimiento, rendimiento de oxidación de sustrato, velocidad máxima de consumo de oxígeno, constante de afinidad y coeficiente de transferencia de masa. La respirometría de pulsos *in situ* demostró ser una técnica reproducible que permitió la determinación de los parámetros cinéticos y estequiométricos además de detectar limitaciones de transferencia de masa en el biorreactor airlift, sin embargo su uso en la oxidación biológica de sulfuro se limita a unos pocos experimentos y condiciones experimentales con una importante inversión de tiempo. Por otro lado, la microrrespirometría de pulsos *ex situ* permitió la adquisición de una mayor cantidad de información en un intervalo más amplio de concentraciones de sulfuro (de 5 a 60 mg H_2S L^{-1}) y condiciones experimentales como diferentes valores de pH. Los resultados obtenidos mostraron que la técnica de microrrespirometría *ex situ* es preferible a la de respirometría de pulsos *in situ* para la caracterización adecuada y confiable del proceso de oxidación de sulfuro.

Palabras clave: Microrespirometría de pulsos, sulfuro oxidación, biorreactor airlift, microbiorreactor, modelos de inhibición.

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https://doi.org/10.24275/rmiq/IA2575
ISSN:1665-2738, *issn-e*: 2395-8472

1 Introduction

Sulfur is one of the most abundant elements on Earth and occurs at different oxidation states (from -2 to +6). Sulfur occurs in form of iron sulfides, mainly pyrite (FeS_2), gypsum ($CaSO_4$) or as sulfate (SO_4^{2-}) (Sánchez-Andrea *et al*., 2014). The $H_2S_{(g)}$ (sulfide) and its ionic forms (HS^- and S^{2-}) are present in natural waters, except for special sulfurous mineral springs.(Pokorna & Zabranska, 2015) The sulfide is a colorless, corrosive, toxic gas with a rotten-egg-like smell. (Cisneros de la Cueva *et al*., 2021) The sulfide and other compounds of anthropogenic origin can significantly pollute the environment and pose a strongly negative impact in many ways. Some sources of these compounds that affect the environment can be for example, acid rain, odor problems from polluted surface water bodies, wastewater treatment plants, sewage, landfills, corrosion of steel and concrete structures, leaching and migration of heavy metals and acidic components of sediments etc.(Haydar & Aziz, 2009; Vannini *et al*., 2008) Accordingly, the sulfide elimination is necessary for many reasons related to health and safety.(Almomani *et al*., 2016; Park *et al*., 2005).

The classical sulfide physico-chemical elimination processes present drawbacK_S, such as large energy requirements and high capital and operating cost (Abdel-Monaem Zytoon *et al*., 2014). The design of biotechnological systems with the purpose of eliminating the sulfide and its ionic forms presents many advantages, for example, low consumption of energy and low cost of implementation (Velasco *et al*., 2019, Loreto Muñoz *et al*., 2021). The elimination of sulfide has been reported to occur in aerobic and anaerobic bioreactors with mechanic and pneumatic stirring systems and removal efficiencies from 50% to 99% (Bonilla-Blancas *et al*., 2015; Zhang *et al*., 2018). During the aerobic biodegradation of sulfide, air is supplied to the bioreactor to carry out either partial or full oxidation of the feeding sulfide according to Eq. 1 and 2 respectively.

$$H_2S + 1/2O_2 \rightarrow S^0 + H_2O \quad (1)$$

$$H_2S + 2O_2 \rightarrow SO_4^{2-} + 2H^+ \quad (2)$$

When the bioreactor is operated with suspended cells it is preferable to carry out partial oxidation to elemental sulfur (Quijano *et al*., 2021), however when the bioreactor is operated with attached cells (e.g. Biotricliking filters) is preferable to carry out full oxidation to avoid bioreactor clogging (Mora *et al*., 2016).

The sulfide removal efficiency depends on several factors, for example, type of substrate (oxidation state), microorganism, type of bioreactor, rheology, stoichiometric and kinetic parameters and pH (Jaber *et al*., 2016; Janssen *et al*., 1995; Zhuo *et al*., 2019).

The bioprocesses designed for the elimination of sulfide require a reliable description of the kinetic and stoichiometric parameters for the proper utilization of mathematical models (Gonzalez-Sanchez *et al*., 2009; Mora *et al*., 2015). The development of mathematical models is necessary to design and optimize the biological removal of sulfide (Mora *et al*., 2016). On the other hand, it is necessary to obtain experimental data from sulfide removal, e.g., kinetics and stoichiometric parameters for a reliable validation of the mathematical model. However, in order to obtain the required parameters is necessary to employ different experimental techniques such as invasive and non-invasive. Therefore, non-invasive methods are preferable, such as influent and effluent mass balance. An alternate non-invasive method could be pulse respirometry, which consists of measuring the dissolved oxygen (DO) after the injection of a defined concentration of substrate into the system (Ordaz *et al*., 2011). Respirometry is defined as the measurement of the exogenous oxygen uptake rate (r_{O2}) in the liquid phase, under well-defined conditions. This technique has been mainly used to estimate kinetic and stoichiometric parameters, e.g. the maximum oxygen uptake rate ($r_{O2,max}$) the substrate affinity (K_S), the substrate oxidation yield ($Y_{O2/S}$) the grow yield ($Y_{x/s}$), the maximum substrate degradation (R_{max}) and the maximum growth rate (μ_{max}) (Espinoza-Rodríguez *et al*., 2012; R. Ramirez-Vargas *et al*., 2013; Vital-Jacome *et al*., 2017). The respirometry is a method that has been successfully proved for biodegradation of various types of substrates such as acetate, phenol, acrylamide, nitrite, ammonium, 4-chlorophenol, glucose, m-cresol methane, sulfur and a mixture of substrates such as actual and synthetic wastewater (Chandran & Smets, 2000; Ellis *et al*., 1996; Mora *et al*., 2016; 2014; Orupõld *et al*., 2001; Vital-Jacome *et al*., 2016). The classical *in situ* pulse respirometry is performed directly into the bioreactor that could be under steady state or non-steady state conditions, for instance a kinetic characterization can be done from the beginning to the end of a bioreactor operation to provide useful information about the evolution of the kinetic and stoichiometric parameters (Ordaz *et al*.,

2011). The technique induces several pseudo steady states characterized by the absence of soluble substrate in which pulses of substrate are added to obtain a DO response, therefore this technique can be used. This characterization often requires a significant amount of experimental effort as well as the characterization of the volumetric mass transfer coefficient of the bioreactor. The number of experiment replicates with *in situ* pulse respirometry is low and also the amount of substrate injected into the system.(Esquivel-Rios *et al*., 2014) An alternative to *in situ* pulse respirometry for the obtainment of the kinetics and stoichiometric parameters is to utilize *ex situ* pulse respirometry that is carried out in microbioreactors, that is *ex situ* pulse microrespirometry. The advantages of the utilization of microbioreactors include the possibility to operate several simultaneous cultures and different experimental conditions in a small space using a continuously shaken set of 24-wells (Kensy *et al*., 2005). The microreactor arrays usually include DO sensors, providing a convenient tool for simultaneous respirometry experiments.

This article aims to apply for the first time the pulse respirometry technique under two different approaches, *in situ* pulse respirometry and *ex situ* pulse microrespirometry, during the biological sulfur oxidation process. These approaches will allow the estimation of the following: (i) a determination of the parameters that can be efficiently retrieved from this technique and (ii) the effect of the substrate concentration and pH on the kinetic and stoichiometric parameters. This first approach to this system studied the reproducibility of the method as well as the drawbacks that could arise.

2 Material and methods

2.1 Experimental Strategy

2.1.1 Airlift-bioreactor set-up

The bacterial culture used in this work was taken from a previously adapted sulfidogenic sludge to aerobic sulfide oxidizing conditions. For that purpose, a bubble column bioreactor inoculated with sulfidogenic sludge from a UASB bioreactor was operated under fed batch mode for one year. The feeding medium contained (g L^{-1}): K_2HPO_4 (0.4), NaCl (1), $CaCl2.2H_2O$ (0.04), $MnCl_2{\cdot}4H_2O$ (0.117), NH_4Cl (0.4), $NaHCO_3$ (0.699) and $Na_2S{\cdot}9H_2O$ (0.741). After that period of time, the adapted sulfide oxidizing culture was used to inoculate an airlift bioreactor which consisted of a glass column of 3 L working volume (0.5 m height and 0.1 m inner diameter) equipped with a draft tube (0.4 m height and 0.004 m inner diameter). The airlift bioreactor was operated during 3 months under fed batch mode at room temperature (25ºC), air supply was maintained at 1.5 L min^{-1} and the pH was controlled at 6.5. The feeding medium for the airlift bioreactor was the same than the previously used in the bubble column, the loading rate of the H_2S fed to the airlift bioreactor was approximately 100 mg L^{-1} d^{-1}. The reactor was equipped with a DO optic measurement system (Flurometrix, Stow MA 01775) linked to a computer for data acquisition in real time.

2.2 Microreactor system

The microreactor system consisted of an unbaffled 24-well microflask system ($OxoDisH_24$, PreSens, México). Each well (16 mm diameter, 18 mm depth and 3 mL total volume) was equipped with a pre-calibrated fluorometric dissolved oxygen (CO_2) sensor (OxoDish, PreSens, Mexico). The unbaffled 24-well microflask system was set on a 24-channel Sensor Dish Reader (SDR-281, PreSens, Mexico) for C_{O2} reading, a personal computer was connected to the entire microreactor system for data acquisition using the PreSens software (SDR v37). The oxygen was provided by superficial aeration from the air present in the headspace of each well, for that purpose, the whole system was set into an orbital shaker at 150 rpm with temperature control at 25 ºC. In order to improve mixing and mass transfer, a glass bead (5 mm diameter) was introduced into each well.

2.3 Respirometric characterization of the biological sulfide oxidation and data interpretation

During the characterization of the kinetic and stoichiometric parameters of the biological sulfide oxidation process, the following considerations and assumptions were taken into account to simplify the microrespirometric data interpretation: (i) biological sulfur oxidation was carried out in one single step, therefore the final product obtained during a microrespirometric experiment was sulfate (SO_4^{-2}). This is in agreement with other authors (Gonzalez-Sanchez *et al*., 2009). (ii) According to the non-structural model for biomass, it was considered that both, sulfur (S^0) and SO_4^{2-} were produced by the same

microorganism. (iii) The chemical oxidation of sulfide in the acid medium can be neglected compared to biological oxidation (Chen & Morris, 1972; Janssen *et al*., 1995). (iv) The formation of SO_3^{2-} and $S_2O_3^{2-}$ under acidic conditions can be neglected (Gonzalez-Sanchez *et al*., 2009; Janssen *et al*., 1995). (v) The polysulfides (S_X^{2-}) production is only possible in an alkaline medium (pH> 9) (Kleinjan *et al*., 2005) and this work was conducted at a maximum pH value of 8, and (vi) according to the low concentrations of pulses injected and the time-lapse during a respirometric assay, the biomass growth can be neglected.

The respirometry technique was applied in both systems, the airlift bioreactor (*in situ* pulse respirometry) and microreactors system (*ex situ* pulse respirometry), with some differences in each case that are described as follows. The first system that was tested was the airlift bioreactor, since it was under fed-batch conditions, the *in situ* pulse respirometry technique was always applied at the end of a given batch. This final stage of a given batch was defined as the point at which soluble sulfide (H_2S) concentration was zero, this is, sulfide was absent. The absence of H_2S was confirmed by analyzing for the H_2S concentration in a sample taken from the airlift bioreactor. Then, the DO system was turned on and once the signal was stabilized, the respirometry protocol was started. First, an aliquot of a stock solution of sulfide that was prepared previously at a concentration of 4600 mg L^{-1} (as $Na_2S{\cdot}9H_2O$), was injected into the airlift bioreactor. The volume of this aliquot was varied in order to have different concentrations of sulfide in the airlift bioreactor: 5, 10, 15 and 20 mg H_2S L^{-1} for a proper characterization. Second, the DO concentration was registered, and the sulfide consumption for a given aliquot injected was followed in a DO concentration chart online (respirogram) as it will be shown in the Results and Discussion section. Finally, the estimation of the volumetric oxygen mass transfer coefficient (K_La) was calculated during each experiment for proper DO data interpretation (Esquivel-Rios *et al*., 2014). The K_La of the airlift bioreactor as well as the K_La of the microreactors were estimated in absence of sulfide following Badino *et al*., (2000) methodology in absence of sulfide as follows: (i) N_2 was sparged into the airlift bioreactor or microreactor system until more than 90% of the DO was removed. (ii) N_2 flow was stopped and the DO was increased until it reached the same previous value (C_b), (iii) the DO data were used to adjust Eq. 3 for K_La determination using the AQUASIM software (Swiss Federal Institute for Environmental Science and Technology Dübendorf, Switzerland). This procedure was done in duplicates for each experiment.

$$\frac{dC}{dt} = K_La \cdot (C_b - C) \tag{3}$$

In the case of the respirometry technique applied to the microreactor system i.e., *ex situ* pulse microrespirometry, three biomass samples were obtained from the airlift bioreactor at three different days. Each sample was used to carry out a microrespirometric characterization at different pH: 6.5, 7, and 8. In order to prepare the biomass samples for microrespirometric characterization, a concentrated biomass solution was prepared as follows: 1000 mL of biomass was collected from the bubble column reactor and centrifuged at 150 rpm and 25 ºC for 10 min (Eppendorf AG, Germany). The supernatant was removed, and the remaining biomass was mixed with 100 mL of mineral medium without $Na_2S{\cdot}9H_2O$ and a buffer solution to maintain the pH at 6.5, 7 or 8. The buffer solution employed was composed of a solution A that contained 278 g L^{-1} of NaH_2PO_4 and a solution B that contained 536.5 g L^{-1} of Na_2HPO_4 $7H_2O$. Both solutions were combined to obtain a final pH of 6.5, 7 or 8 according to method (Macdonald, 2010). The volatile suspended solids (VSS) content of the biomass solution was of 1150 ± 353.553 mg VSS L^{-1}.

During each microrespirometric characterization, the concentration of sulfide used was varied, with that purpose, several stock solutions of sulfide were prepared by adding 1150, 2300, 3000, 4600, 6960, 10440 and 13990 mg L^{-1} of $Na_2S{\cdot}9H_2O$. During the *ex situ* pulse microrespirometric experiments, the stock solutions were diluted to the desired final concentration in the microreactors, which were of 5, 10, 15, 20, 30, 45, and 60 mg L^{-1} of sulfide. Briefly, the *ex situ* pulse microrespirometric protocol consisted of the following steps: (i) preparation of the biomass stock solution and sulfide stock solution described above. (ii) each well of the microreactor system was filled with 1.45 mL of the biomass stock solution. (iii) the DO was monitored until a stable signal was obtained. (iv) determination of the K_La. (v) each well was added with a pulse of 0.05 mL of the diluted sulfide stock solution corresponding to different concentrations: 5, 10, 15, 20, 30, 45, and 60 mg L^{-1}, each concentration was tested in triplicate including a control experiment in which no sulfide was added. (vi) the DO response was followed and registered until steady state conditions were reached

again and (vii) the K_La was determined again.

The autotrophic growth of sulfide oxidizing bacteria can be expressed according to Eq 4.

$$\alpha H_2S + \beta O_2 + \gamma CO_2 \rightarrow \delta C_5H_7NO_2 + \varepsilon SO_4^{2-} + \zeta H^+ + \eta H_2O \quad (4)$$

If reagents and products are expressed in chemical oxygen demand (COD) units (the amount of oxygen needed for their complete oxidation), only sulfide, oxygen and biomass have to be considered and an oxygen mass balance can be written as follows.

$$\alpha 64 - \beta 32 = \delta 160 \quad (5)$$

Where 64 is the mass of oxygen (g) needed to oxidize 1 mole of H_2S and 160 is the amount of oxygen (g) needed to oxidize 1 mole of biomass. By rearranging Equation 5 we obtain:

$$\frac{\beta 32}{\alpha 64} + \frac{\delta 160}{\alpha 64} = 1 \quad (6)$$

The first term represents the mass of oxygen consumed per unit of sulfide consumed, that is substrate oxidation yield ($Y_{O2/S}$). The second term represents the mass of biomass produced per mass of sulfide consumed, that is the biomass growth yield ($Y_{X/S}$).

After a given sulfide pulse was injected, whether in the airlift bioreactor or in the microreactor system, a DO mass balance in each well of the microreactor system could be calculated by using Eq. 7 in which a balance between the oxygen uptake rate (r_{O2}) and the DO provided by the continuous aeration was done.

$$\frac{dC}{dt} = K_La \cdot (C_b - C) - r_{O2} \quad (7)$$

The $Y_{O2/S}$ can be first estimated from the amount of oxygen consumed during a pulse injection of sulfide (S_p), then $Y_{X/S}$ can be obtained following Eq. 8

$$Y_{X/S} = 1 - Y_{O2/S} = 1 - \frac{\int_0^t r_{O2}dt}{S_p} = 1 - \frac{k_La \cdot \int_0^t (C_b - C)dt + (C_0 - C_f)}{S_p} \quad (8)$$

Where C_b is the baseline DO concentration, C_0 and C_f are the initial and final DO concentrations, respectively.

During a given sulfide pulse injection, the maximum oxygen uptake rate observed ($r_{O2'max}$) was determined from the average of the ten highest values of the respiration rate observed at that concentration of pulse. It was observed that the $r_{O2'max}$ data obtained during each experiment in the microreactor system followed either a Monod behavior (*in situ* pulse respirometry) or a substrate inhibitory behavior (*ex situ* pulse microrespirometry), as it will be shown in the Results and Discussion section. For that reason, the $r_{O2'max}$ data obtained in each experiment were interpreted taking into account the Monod model or several mathematical models commonly used during substrate inhibitory processes. The models tested in this study were Haldane, Aiba, Edwards and Andrews (Table 1).

All the results obtained were submitted to an analysis of variance (ANOVA) and Tukey-Kramer tests that were performed with NCSS® statistical software.

Table 1. Biological models tested in this study.

Model	r_{O2} =
Monod	$r_{O2max} \cdot \frac{S}{K_s+S}$
Andrews	$r_{O2max} \cdot \frac{S}{K_s+S+\frac{S^2}{K_I}}$
Aiba	$r_{O2max} \cdot \frac{S}{K_S+S} \cdot e^{\left(-\frac{S}{K_I}\right)}$
Edwards	$r_{O2max} \cdot \left(e^{\left(-\frac{S}{K_I}\right)} - e^{\left(-\frac{S}{K_s}\right)}\right)$
Haldane	$r_{O2max} \cdot \frac{S}{(S+K_s)\cdot\left(\frac{1+S}{K_I}\right)}$

Where, r_{O2}, Specific oxygen uptake rate (mg O_2 L^{-1} h^{-1}), r_{O2max}, Maximum exogenous oxygen uptake rate (mg O_2 L^{-1} h^{-1}), K_s Substrate affinity constant (mg H_2S L^{-1}), K_i, Substrate inhibitory constant (mg H_2S L^{-1}).

2.4 *Sulfide stripping estimation*

The sulfide stripping in the airlift bioreactor and in the microreactors was theoretically calculated by a mass balance in the gas (Equation 9), and liquid (Equation 10) phases, as described by Gonzalez-Sanchez *et al.*, (2009).

$$\frac{dH_2S_{(g)}}{dt} = \frac{V_L}{V_G} * K_L a_{H2S} * \left[\left(\frac{H_2S_{(l)}}{1+10^{(pH-pK1)}+10^{(2*pH-(pK1-pK2)}}\right) - \frac{H_2S_{(g)}}{He}\right] - \left(\frac{Q_{air}}{V_G} * H_2S_{(g)}\right)$$

$$t = 0; \quad H_2S_{(g)} = 0 \tag{9}$$

$$\frac{dH_2S_{(L)}}{dt} = -\left[1+10^{(pH-pK1)}+10^{(2*pH-(pK1-pK2))}\right] * K_L a_{H2S} * \left[\left(\frac{H_2S_{(L)}}{1+10^{(pH-pK1)}+10^{(2*pH-(pK1-pK2))}}\right) - \frac{H_2S_{(G)}}{He}\right]$$

$$t = 0; \quad H_2S_{(L)} = S_P \tag{10}$$

Where pK_1 (7.1) and pK_2 (13.6) are the dissociation constants for sulfide; He is the Henry constant for sulfide (0.41 (mmol L^{-1})$_G$/(mmol L^{-1})$_L$); V_L is the liquid volume of either the airlift bioreactor or the microreactor; V_G is the gaseous headspace volume of either the airlift bioreactor or the microreactor; $H_2S_{(L)}$ and $H_2S_{(G)}$cc are the sulfide concentration in liquid and gas, respectively.

In the case of the microreactors system the airflow rate (Q_{air}) was set to a value of zero since the aeration was only provided by orbital agitation. The volumetric sulfide mass transfer coefficient ($K_L a_{H2S}$) was estimated from the value of $K_L a_{O2}$ taking into account the pH value of the liquid medium (Equation 11) as described by Yongsiri *et al*., (2003).

$$K_L a_{H2S} = K_L a_{O2} \cdot (1.736 - 0.196 \cdot pH) \tag{11}$$

3 Analytical methods

For the experiments in which a determination of sulfur (S^0) was carried out, these determinations were determined by a spectrophotometric method. The microorganisms have the capability of forming deposits intracellular and extracellularly of sulfur globules (Cerri *et al*., 2016). Based on bibliographic information we can consider that the microorganisms culture contained the two types of bacteria. After each respirometric assay in the airlift bioreactor, it was taken 20 mL of sample and 5 mL was deposited in a test tube, after that, the samples were filled up with 5 mL of ethanol 96% (Van Gemerden, 1968). The samples were dried at 60°C, subsequently, the dried samples were grind up and weighed, with the aim to compare if each sample was homogeneous. Immediately, a volume of 5 mL of $CHCl_3$ was added to each sample and it was refrigerated during 18-24 h at 7°C. A dilution (1:5 V/V) was required before reading in a UV - visible spectrophotometer (Perkin Elmer), with a 1 cm thick quartz cell of pass and at a wavelength (λ) of 290 nm. The calibration factor for sulfur was calculated from the absorbance of solutions of re-sublimated sulfur in $CHCl_3$. Sulfur concentrations are expressed in mg L^{-1}.

The sulfate (SO_4^{2-}) was analyzed according to standard methods according to Guerrero-Barajas *et al*., (2015). Here, the quantification of sulfate as barium sulfate is done by using a turbidimetric method. The sample was placed in 5 mL of a conditioning solution (hydrochloric acid HCl 1:1) in a volumetric flask of 25 mL, addition of 1 mL of the previously centrifuged sample (at 11,320 × g), is completed to the 25 mL of the volumetric flask with distilled water and 1 g of barium chloride was added. The solution was mixed for 1 min in a vortex. After 4 min, the barium sulfate is formed and then the sample can be read in the spectrophotometer at a wavelength (λ) of 420 nm (APHA *et al*., 2017).

Biomass concentration in the airlift bioreactor was determined at the beginning and at the end of the experiments and reported as VSS content (mg L^{-1}) according to literature (APHA *et al*., 2017).

4 Results and discussion

4.1 Airlift bioreactor operation

Figure 1 shows the concentrations profile of the chemical species and the biomass followed during five months in the airlift bioreactor. From the Figure 1 it can be seen that sulfide (H_2S) loading fed to the airlift bioreactor was constant at a value of 94.56 ± 5.03 mg H_2S $d^{-1}L^{-1}$ during the five months, also, the biomass concentration was maintained at 6.07 ± 0.61 g VSS L^{-1} after 100 days of operation. During the entire experiment it was observed an accumulation of S^0 in the airlift bioreactor which was periodically removed and as it can be seen in the Figure 1, the So concentration varied between 30 and 68 mg L^{-1} with an average value of 44.97 mg L^{-1}. On the other hand, sulfate (SO_4^{2-}), which is the oxidized form of H_2S remained at low concentration values during the

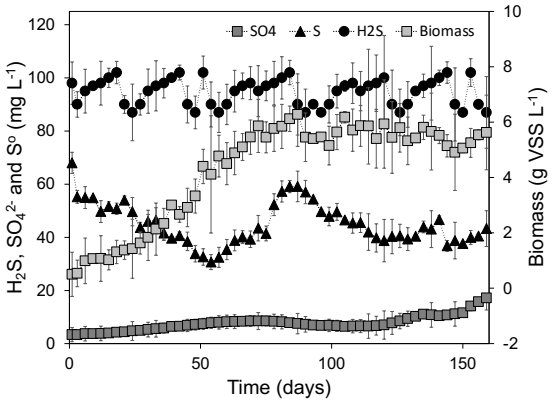

Figure 1. Chemical species and biomass concentration observed during the three months operation in the airlift bioreactor.

beginning of the operation of the airlift bioreactor and it was observed a slightly increase after 40 days to reach an average value of 12.31 ± 2.49 mg SO_4^{2-} L^{-1}. The low conversion to SO_4^{2-} may be attributed to the low dissolved oxygen concentration (less than 1 mg L^{-1}) provoked by the low airflow rate. Janssen *et al*., (1995) and Stefess *et al*., (1996) also reported a 50 % and 60% of the conversion from SO_4^{2-} to S^0, respectively attributed to the low DO concentration (less than 1 mg L^{-1}). The difference between the sulfide that was fed, and the sulfur removed in the form of S^0 and SO_4^{2-} could be attributed to volatilization of sulfide, which occurred within a range of 25 - 49%. As it will be discussed ahead, during the theoretical analysis of stripping rate of the airlift bioreactor, more than a half of the initial sulfide concentration added to the bioreactor could be stripped to the atmosphere during the first hour after its addition. The bioreactor was operated under fed-batch mode and the feeding that was done once per day, hence an important sulfide volatilization might have been happening just after the feeding.

4.1.1 In situ pulse respirometry experiments

After 40 days of airlift bioreactor operation, the *in situ* pulse respirometry protocol was started for the kinetic and stoichiometric characterization of the sulfide oxidation process. On days 6, 9, 12, and 15, pulses of H_2S of 5, 10, 15, and 20 mg L^{-1} were injected in a row each day. The results shown in Figure 2 indicate that the pulses were highly reproducible along these days.

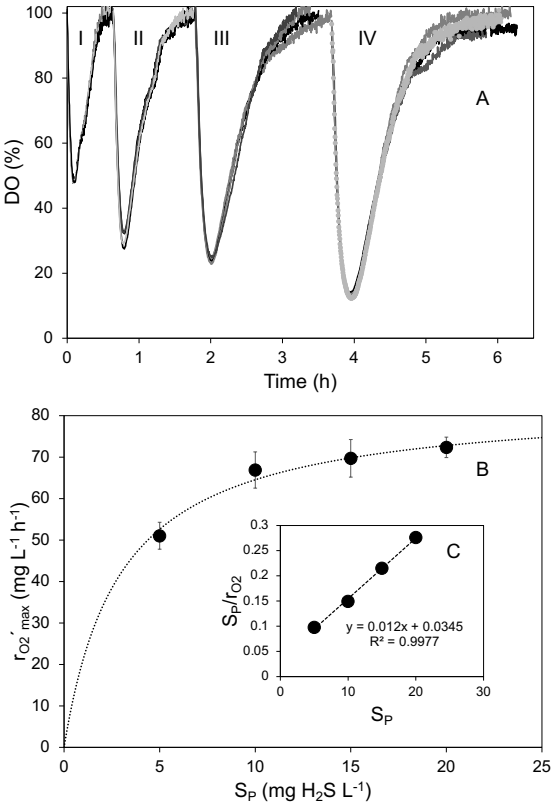

Figure 2: (A) Reproducibility of the sulfide pulses injected in 4 different days (days 6, 9, 12 and 15), (B) Example of a Monod curve obtained using the $r_{O2'max}$ values observed after injections of increasing sulfide concentration pulses, and (C) Hannes Woolf linearization of plot B.

$Y_{O2/S}$ and $Y_{X/S}$ were the first parameters being estimated and it was observed that the $Y_{O2/S}$ increased its value as S_P increased. Hence, the $Y_{O2/S}$ value ranged between 0.486 to 0.710, being significantly higher at S_P of 15 and 20 mg H_2S L^{-1} compared to S_P of 5 mg H_2S L^{-1}. Accordingly, the $Y_{X/S}$ decreased its value as S_P increased and it ranged between 0.514 - 0.290, these values were slightly high taking into consideration the autotrophic growth of sulfide oxidizing bacteria, which typically ranged between 0.11 - 0.2 μ_m h^{-1} for genera of bacteria such as *Pseudomonas* and *Thiobacillus* (Xu *et al*., 2016). This result was unexpected since previous reports regarding the stoichiometric parameters ($Y_{X/S}$ and $Y_{O2/S}$) pointed out that no significant difference was observed at different S_P values and even under different hydrodynamic conditions tested during the respirometric experiments (Ordaz *et al*., 2019).

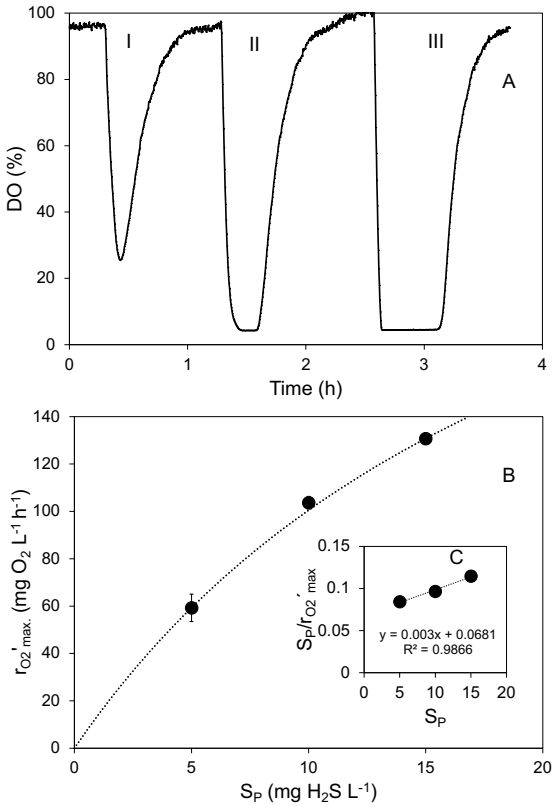

Figure 3: (A) Respirogram from the airlift bioreactor obtained on day 100, (B) Monod curve obtained using the $r_{O2'max}$ values observed after injections of increasing sulfide concentration pulses, and (C) Hannes Woolf linearization of plot B.

The high values obtained for $Y_{X/S}$ and $Y_{O2/S}$ indicates that these parameters might be affected by other factor such as sulfide stripping that avoids the correct estimation of the sulfide pulse added to the bioreactor, this will be discussed below. On the other hand, as it is shown in Figure 2B, the DO curves obtained during those respirometric experiments allowed the determination of the $r_{O2'max}$ in each pulse, the values obtained followed a Monod behavior. The r_{O2max} and the K_S average values determined in those respirometric experiments were of 83.48 ± 5.58 mg O_2 g^{-1} VSS h^{-1} and 2.94 ± 0.57 mg H_2S L^{-1}, respectively.

After 100 days of operation, it was observed a steady state in the value of biomass concentration (see Figure 1) and the *in situ* pulse respirometric experiments were repeated in order to evaluate the evolution on the kinetic and stoichiometric parameters. Figure 3A shows that the injection of the same concentrations of H_2S provokes a sharp decrease in the DO saturation that was critical at concentrations above 10 mg H_2S L^{-1} in which the DO saturation reached values close to zero. It was decided not to inject a higher concentration beyond 15 mg H_2S L^{-1} since a clear DO limitation was observed. Under these conditions, the values of $Y_{O_2/S}$ and $Y_{X/S}$ were of 0.399 ± 0.038 and 0.601 ± 0.039, respectively and not significant difference was observed between the values obtained at different S_P. On the other hand, an important increase in the r_{O2max} and the K_S average values occurred on day 100, which were of 329.55 ± 37 mg O_2 L^{-1} h^{-1} and 19.21 ± 2.34 mg H_2S L^{-1}, respectively. This increase in the kinetic activity was related to the increase of biomass concentration that on day 100 was 500 % superior to the biomass concentration observed during the first respirometric experiment (day 6 to 15). The K_La measured in the airlift at this period was of 11.66 ± 0.87 h^{-1}, that corresponded to an OTR_{max} of 71.5 ± 1.28 mg L^{-1} h^{-1}. This value of OTR_{max} was clearly inferior to the r_{O2} observed during the respirometric experiments, therefore the flat curve observed when the DO reaches the lowest values indicated an oxygen transfer problem in the airlift bioreactor. Therefore, the low oxygen mass transfer conditions did not tie the high r_{O2} activity observed due to the increase in biomass concentration attained in the bioreactor and a DO limitation was observed. Under these conditions the kinetic parameters estimation was probably hindered.

An airflow rate of 1.5 L min^{-1} was used during all *in situ* respirometric experiments, this value allowed the suspension of solids, with a lower airflow rate it was observed some precipitation of solids. Hence, the K_La measured in the airlift along the two respirometric experiments was in a range of 5.68 ± 0.15 h^{-1} (first experiment) and 11.66 ± 0.87 h^{-1} (second experiment). Other studies have also reported low mass transfer conditions during biological sulfide oxidation, for instance, Mora *et al.*, (2015) reported K_La values ranging from 6 to 9 h^{-1}. These low oxygen mass transfer conditions favored the formation of S^0, which is preferable over the SO_4^{-2} formation due to the energy consumption for aeration, also, these conditions provoked a lower sulfide stripping into the environment. Figure 4A shows the theoretical stripping observed into the airlift bioreactor when pulses of different concentration of sulfide were injected, these curves corresponded to stripping rates in a range of 10.63 - 42.56 mg H_2S L^{-1} h^{-1}, thus the sulfide stripping rate increased as the S_P increased.

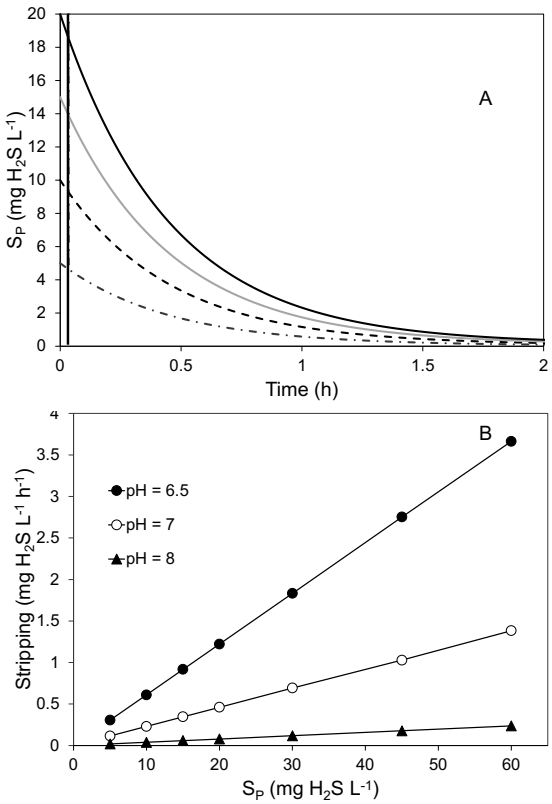

Figure 4. Sulfide stripping curves calculated with Equations 4 and 5. (A) Sulfide concentration profiles calculated at different concentrations of sulfide injected into the Airlift bioreactor and (B) Stripping rate calculated at different pH and sulfide concentrations in the microreactors.

The stripping of sulfide was of high interest since it might have been affecting the proper estimation of the stoichiometric parameters $Y_{O2/S}$ and $Y_{X/S}$ according to the protocol of *in situ* pulse respirometry. Figure 2 and 3 showed that a given *in situ* pulse respirometric experiment lasted between 0.5 h and 1 h and according to Figure 4A, the 10% of the initial sulfide injected was stripped within the first 3 minutes, and 50% after 19 minutes, this indicates that the actual amount of sulfide oxidized was less than the sulfide added to the bioreactor which provoked a low oxygen consumption. Under these conditions, the kinetic parameters, i.e., r_{O2max} and K_S, that are usually determined within the first 3 minutes of the respirometric experiment might be determined with a lower error, however the low oxygen mass transfer represents a drawback of this operating conditions.

According to the results obtained, four important aspects can be discussed for the application of *in situ* respirometry to the sulfide oxidation airlift bioreactor as it was typically done in other studies: (i) since a significant sulfide stripping was occurring during *in situ* respirometric experiments even at low air flow rate, a new configuration of the airlift bioreactor would be helpful such as the use of a closed bioreactor equipped with a small compressor that recirculates the gas in the headspace to the bioreactor as it was done during methane oxidation studies by our group (Ordaz *et al*., 2014), (ii) the total time for a complete respirometric assay, including the injection of up to 4 pulses, was almost 6 h (without including duplicates of each pulse). This time does not include the determination of K_La that accounts for almost 3 h, hence a total time of 9 h was needed for each respirometric assay that includes only the single injection of 4 different pulses of H_2S (S_P). This is a considerable amount of experimental time, also this span of time might originate changes in the microbial metabolism, which in turn may affect the $Y_{X/S}$ and $Y_{O2/S}$ estimation at different S_P values as it was observed in the first experiment. (iii) The high r_{O2} activity observed as the biomass concentration increased along with the poor oxygen mass transfer condition in the airlift bioreactor might affect the correct estimation of kinetic parameters and, (iv) the low sulfide stripping condition is preferable during the estimation of kinetic and stoichiometric parameters. These three issues were taken into account during the implementation of the *ex situ* pulse microrespirometry experiments during a second stage.

4.2 *Ex situ microrespirometric experiments*

The microreactors used in this stage of the study have been successfully used in other studies (Esquivel-Rios *et al*., 2014; Hernández-Martínez *et al*., 2018; Rocio Ramirez-Vargas *et al*., 2014; Vital-Jacome *et al*., 2016, 2017). Therefore, their utilization during this stage of our study was mainly focused on: (i) testing more experimental conditions using pulse respirometry involving less experimental time, i.e., to study a broader range of H_2S concentrations, and the effect of pH on the kinetics and stoichiometry of sulfide removal. (ii) the maintenance of similar oxygen mass transfer and low sulfide stripping conditions in the microreactors avoiding a high r_{O2} activity by diluting the biomass concentration present in the airlift bioreactor.

In regards to sulfide stripping analysis in the microreactors, Figure 4B shows that the theoretical sulfide stripping rate was decreasing as the pH

increased due to the dissociation of sulfide and also the stripping rate increased with the sulfide concentration. Additionally, the values of the stripping rate were much lower in the microreactors than in the airlift bioreactor mainly due to the substitution of the airflow by an aeration surface provided by the orbital stirring. Yongsiri *et al*., (2003) pointed out that the stripping of sulfide was mainly influenced by the turbulence in the system as well as the pH. Since our system was maintained at the same conditions of orbital stirring (150 rpm), the pH was the only factor that played a significant role during the sulfide stripping process in the microreactors.

Unlike the previous experiments in the airlift reactor, during the *ex situ* pulse microrespirometric experiments it was possible to inject a total of seven different pulses of H_2S with concentrations higher than the pulses injected to the airlift bioreactor during the *in situ* pulse respirometry. This procedure resulted in a better insight on the kinetic analysis of the H_2S removal. Moreover, the kinetic parameters were analyzed under three different pH values with a single sample taken from the airlift bioreactor on day 120, and the experiments were repeated with another two samples taken from days 125 and 130. Complete *ex situ* pulse microrespirometric for a given sample was done only once and the total experimental time was 3 h, which represents a minimum experimental effort compared with the *in situ* pulse respirometric method applied to the airlift bioreactor.

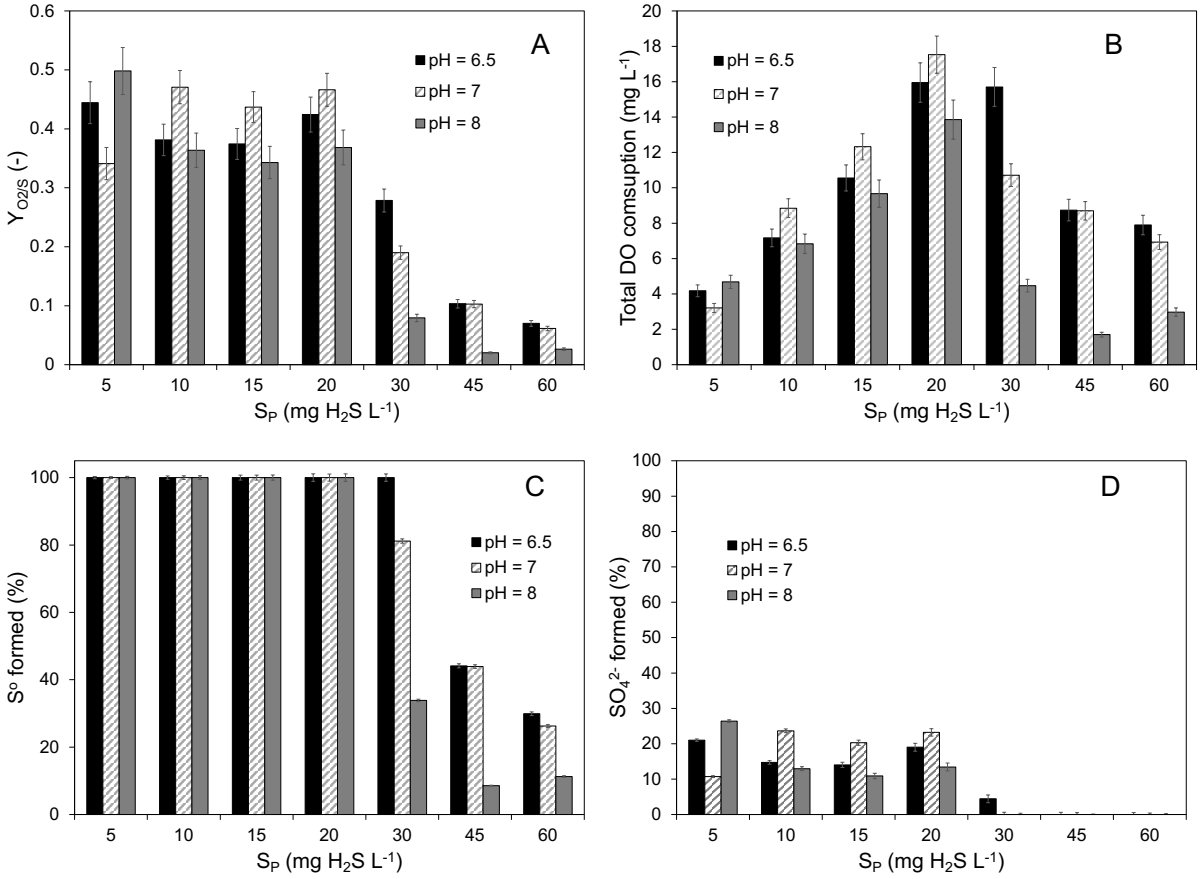

Figure 5. (A) $Y_{O2/S}$ for H_2S oxidation to SO_4^{2-}, (B) DO consumption observed during microrespirometric experiments, (C) Theoretical S^0 formation with the total DO consumption observed and (D) Theoretical SO_4^{2-} formation with the total DO consumption observed.

First, the stoichiometric and kinetic parameters were estimated from respirometric experiments following the same experimental protocol with a special emphasis in the stoichiometric consumption of DO (Figure 5). Figure 5A shows that the $Y_{O2/S}$ for H_2S oxidation to SO_4^{2-} was in a range of 0.50 - 0.02, being significantly lower at higher concentrations of H_2S. This $Y_{O2/S}$ led to a higher $Y_{X/S}$ which was in a range of 0.50 - 0.98, a yield that is biologically difficult to achieve given the autotrophic nature of sulfide oxidizing bacteria, therefore it was assumed that accumulation of H_2S was occurring in the cases of pulses higher than 20 mg H_2S L^{-1}. Figure 5B shows the total DO consumption measured during the microrespirometric experiments, it was observed a continuous increase of the DO consumption during the injections of pulses of 5 to 20 mg H_2S L^{-1}, whereas at higher H_2S pulses the DO consumption diminished drastically, the same behavior was observed when the pH was varied from 6.5 to 8. It is important to note that the minimum DO concentration attained was 1.6 mg L^{-1} in the case of pulses between 5 to 20 mg H_2S L^{-1} whereas in the cases of the injected pulses of 30 to 60 mg H_2S L^{-1} the minimum DO concentration remained between 1.6 and 4.8 mg L^{-1}. As it will be discussed ahead, there is an inhibitory effect observed at high concentrations of H_2S and this is in correlation with a low DO consumption and a high DO concentration that did not correspond with the total amount of the pulse of H_2S injected. The measured values of total oxygen consumption were used to calculate the percentage of S^0 (Figure 5C) and SO_4^{2-} (Figure 5D) that would be produced taking into account the stoichiometry ratio of 0.5 mg O_2 mg^{-1} Sº and 1 mg O_2 mg^{-1} SO_4^{2-} (Equations 1 and 2). According to Figure 5C, the DO consumption measured during injection of pulses from 5 to 20 mg H_2S L^{-1} would be enough to carry out a full stoichiometry oxidation of H_2S to S^0 and further partial oxidation to SO_4^{2-} (Figure 5D). However, the total DO consumption measured during injection of pulses from 30 to 60 mg H_2S L^{-1} would be only enough to carry out a partial oxidation of H_2S to S^0 indicating that not all the H_2S injected in the microreactor was being oxidized. Due to the bioreactor set up and the low volume used (1.5 mL) it was not possible to carry out a sampling procedure for a full study of the chemical species that would be formed during the microrespirometric experiment. Nevertheless, the evidence about not oxygen limitation (DO concentration > 20%) and the total measured DO consumption indicated that a possible inhibitory effect was occurring.

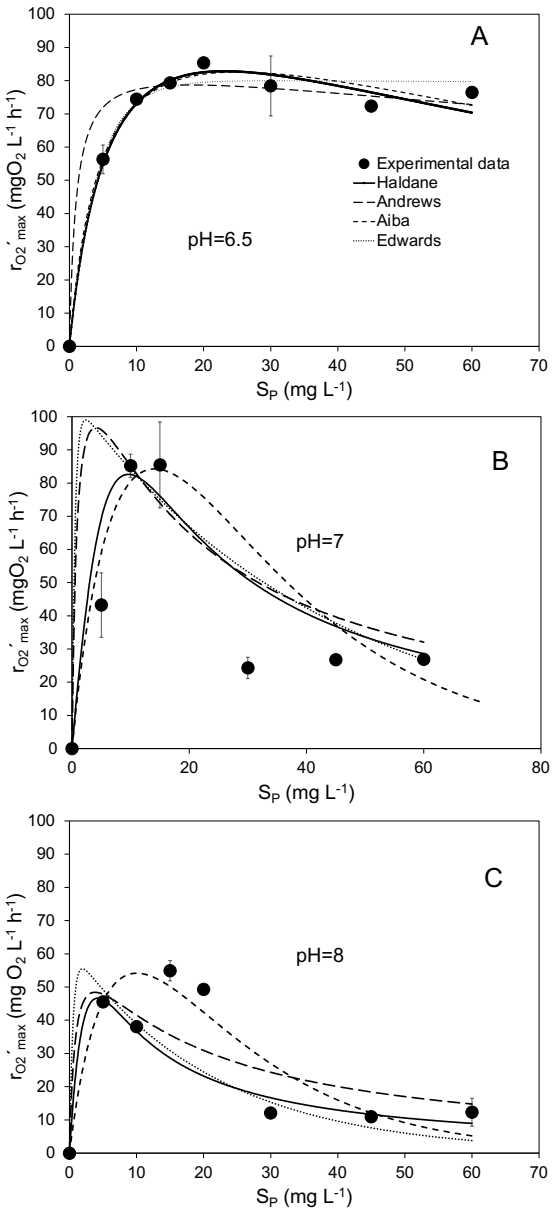

Figure 6. Experimental data (·) observed at different pH and sulfide concentrations and best predictions of Haldane (– – –), Aiba (- - -), Edwards (· · ·) and Andrews (- -) models.

Figure 6 shows that the consumption of H_2S followed an inhibition kinetic behavior that was not possible to observe in the airlift bioreactor due to the low concentrations of the H_2S pulses injected. During the respirometric experiment at pH 6.5 (Figure 6A), which was conducted at the same pH as in the airlift bioreactor, the $r_{O2'max}$ obtained with the injections of higher concentrations than 20 mg H_2S L^{-1} showed a slight reduction in its value that might indicate a

substrate inhibition process. This substrate inhibition effect became stronger as pH increased to values of 7.0 (Figure 6B) and 8.0 (Figure 6C). The inhibition effect on the respiratory activity caused by increasing the pH has also been observed by different authors (Bonilla-Blancas *et al*., 2015), which reported an inhibition effect when pH was superior to 8 however the sulfide concentrations tested were between 0 and 9 mg H_2S L^{-1}. Since in the present work a broader range of H_2S concentrations was tested, the inhibitory effect was observed to occur at pH 7. As it is shown in Figure 4B, the theoretical stripping rate of H_2S decreased as pH increased from 6.5 to 8, indicating that the sulfide oxidation microorganisms were in contact with a higher amount of H_2S during the experiments carried out at pH 7 and 8 compared with the experiments carried out at pH 6.5. Furthermore, according to the theoretical speciation of H_2S at pH 6.5 the sulfur species (S_p) injected were 79.8 % as H_2S, 23.2 % as dissolved sulfide (HS^-), whereas at pH 8 the S_P injected was 9.5 % as H_2S and 90.5 % as HS^-. It has been reported that the HS- is consumed faster than H_2S by the bacteria (Buisman *et al*., 1990), however, to the best of our knowledge there is a lack of literature reports about inhibition of bacteria by a high amount of HS^-. The combination of pH and higher concentrations of H_2S presents a deeper inhibition effect on the biological sulfide oxidation process observed in this study. An explanation of this effect might be as follows: (i) At pH of 6.5 the H_2S stripping rate increases, therefore, a low concentration of H_2S is distributed between H_2S and HS^- concentration from which HS^- concentration is even lower. (ii) As pH increases, the stripping rate decreases and the high concentration of H_2S is highly distributed in the form of HS^-, which might provoke a substrate inhibition effect due to the high concentrations of H_2S injected.

The r_{O2} versus S_P charts obtained at the three different pH values were used to adjust several inhibition models (Table 1) to estimate the kinetic parameters that are shown in Tables 2 to 4. This is the first time that different substrate inhibition models were applied to characterize the biological sulfide oxidation process with *ex situ* microrespirometric data due to feasibility to test a broader range of H_2S concentrations in the microreactors system. For all tested cases, it was determined that the adjustment of the Aiba model to the experimental data was the best as it was indicated by the correlation coefficient which showed average values in a range of 0.858 - 0.989.

Table 2. Kinetic parameters estimated at pH 6.5.

Parameter/ Model	Aiba	Haldane	Andrews	Edwards
r_{O2max} (mg O_2 L^{-1} h^{-1})	117.14 ± 12.78	129.53 ± 12.50	188.82 ± 24.66	80.33 ± 4.62
K_S (mg H_2S L^{-1})	5.03 ± 2.02	6.51 ± 2.35	325.33 ± 74.24	4.09 ± 0.98
K_I (mg H_2S L^{-1})	115.36 ± 49.73	81.85 ± 2.34	151.05 ± 19.73	780.18 ± 56.65
R^2	0.989 ± 0.006	0.988 ± 0.007	0.966 ± 0.008	0.987 ± 0.002

Table 3. Kinetic parameters estimated at pH 7.

Parameter/ Model	Aiba	Haldane	Andrews	Edwards
r_{O2max} (mg O_2 L^{-1} h^{-1})	430.33 ± 129.20	601.58 ± 8.61	755.62 ± 3.07	105.77 ± 2.77
K_S (mg H_2S L^{-1})	24.16 ± 8.72	30.26 ± 0.18	16.71 ± 0.43	0.53 ± 0.03
K_I (mg H_2S L^{-1})	22.30 ± 5.55	3.07 ± 0.10	3.31 ± 0.07	43.75 ± 0.33
R^2	0.858 ± 0.001	0.829 ± 0.014	0.693 ± 0.038	0.713 ± 0.036

Table 4. Kinetic parameters estimated at pH 8.

Parameter/ Model	Aiba	Haldane	Andrews	Edwards
r_{O2max} (mg O_2 L^{-1} h^{-1})	261.34 ± 105.83	149.76 ± 50.04	210.94 ± 81.09	62.14 ± 12.22
K_S (mg H_2S L^{-1})	16.12 ± 8.57	4.67 ± 3.69	14.50 ± 3.48	0.50 ± 0.00
K_I (mg H_2S L^{-1})	16.29 ± 0.17	3.83 ± 1.06	5.31 ± 1.20	21.43 ± 14.38
R^2	0.906 ± 0.024	0.809 ± 0.009	0.846 ± 0.017	0.788 ± 0.134

The respirometric trend that is observed in Figure 6 corresponds to the changes determined in the value of K_I for all the models: as the pH increased, the value of K_I decreases indicating an increase in the inhibitory phenomenon. The inhibition phenomenon studied in the microreactors might be affecting the performance of the bioreactor during its operation under fed batch mode. The feeding of 94.56 ± 5.03 mg H_2S $d^{-1}L^{-1}$ occurred only once a day and the bioreactor was exposed to a high concentration of sulfide that could have been provoking an inhibition phenomenon and eventually a volatilization of the sulfide that was not consumed.

Conclusions

The application of respirometry to determine kinetic and stoichiometric parameters during biological sulfide oxidation has been previously utilized by several authors, however, the utilization of dynamic pulse respirometry applied under two approaches: *in situ* pulse respirometry and *ex situ* pulse microrespirometry, had never been applied. In view of the results presented in this work, it can be concluded that *in situ* pulse respirometry is not suitable to carry out a full characterization of the biological sulfide oxidation process. This is mainly due to the limited number of experiments and the low range of H_2S concentrations than can be conducted and the sulfide stripping phenomenon. On the other hand, during *ex situ* pulse microrespirometry, even though the theoretical stripping rate was diminished, the obtainment of $Y_{O2/S}$ and $Y_{X/S}$ was still uncertain. However, the *ex situ* pulse microrespirometry provided useful information in regards to substrate inhibition at different pH values with the estimation of three important parameters: r_{O2max}, K_S and KI. This is the first time that a double inhibitory effect (H_2S concentration and pH) on the sulfide oxidation process is reported. Undoubtedly, the utilization of microreactors allowed the characterization of different samples taken from the airlift bioreactor under a broader range of H_2S concentrations and pH conditions during short experimental time, which is a better approach for biological sulfide characterization, indicating the potential utilization of microreactors to perform kinetic analysis. However, both respirometry techniques showed a drawback concerning $Y_{O2/S}$ and $Y_{X/S}$, since their respirometric estimation requires to know precisely the amount of oxygen consumed during a pulse injection which was difficult to estimate under sulfide volatilization and substrate inhibition phenomena.

Acknowledgements

We gratefully acknowledge the CONACYT graduate scholarship granted to Gabriel Alejandro Keb - Fonseca (191663) and the financial support provided by Secretaría de Investigación y Posgrado -Instituto Politécnico Nacional (20170311 - 20201126) to Guerrero - Barajas C. The authors also thank Dr. Frederic Thalasso Siret from the Centro de Investigación y Estudios Avanzados, IPN (CINVESTAV) for his technical support. The authors declare that they have no conflict of interest

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