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Modelling of Ferrous Sulphate Oxidation by *Thiobacillus ferrooxidans* in Discontinuous Culture: Influence of Temperature, pH and Agitation Rate

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This paper studies the temperature effect on the specific growth rate of the microorganisms (μ_c) as particular aspect of the mathematical relationships between μ_c of *Thiobacillus ferrooxidans* and the concentrations of substrate (ferrous iron) and product (ferric iron). Until now, there have been few studies of this relationship; also, there has been little work studying the influence of temperature on growth kinetics of this bacteria. In this paper, an extensive experimental phase has been developed, with the aim of determining with precision the influence of temperature on the maximum specific growth rate (μ_{max}). The influence on the growth rate of *T. ferrooxidans* exerted by the initial pH of the medium and by agitation rate has also been studied.

[**Key words:** *Thiobacillus ferrooxidans*, ferrous sulphate oxidation, temperature, pH, kinetic model, agitation]

Biological oxidation of ferrous sulphate can be defined as an aerobic process by which a substrate containing Fe (II) is oxidized, in acid medium, by *Thiobacillus ferrooxidans*, to give Fe (III) compounds. The capacity of this microorganism to obtain energy from the oxidation of ferrous to ferric iron is exploited in various industrial processes, such as: biohydrometallurgy (1), treatment of acid mine drainage (2, 3) and processes for removal of hydrogen sulphide present in many industrial waste gases that cause environmental contamination (4).

The variables with the most influence on the metabolism of *T. ferrooxidans* are: ferrous iron, ferric iron, pH, temperature, dissolved oxygen concentration, carbon dioxide concentration and inert solids. Factors that have been most studied in such metabolism include the effects of substrate and product concentration and both have complex influences on growth. Several authors have reported on the difficulties certain strains of *T. ferrooxidans* experience in growing in media with concentrations of ferrous iron above 5.5 g/*l* (5) and on the severe inhibition likely to result in the case of a Fe (III) concentration of 2.0 g/*l* (6).

There are several kinetic models with predict the effect of the parameters on the specific growth rate of *T. ferrooxidans* (4, 7–10) but it should be noticed that even though the models are quite complex, they do not take account of the influence of temperature. Until now, in the literature, only one general equation which considers the influence of temperature on the growth of microorganisms has been proposed (11).

$$\mu_{max} = A \cdot e^{-E_a/RT} - B \cdot e^{E_b/RT}$$

Where E_a and E_b are the activation energies for cellular multiplication and thermal denaturation processes, respectively, and A and B are the experimental factors. Since the energies activating the biochemical processes cause denaturation are higher than those for multiplication processes, such curves are unsymmetrical in relation to the optimum, and with steeper slope on the right-hand side, with an optimum nearer the maximum temperature than to the minimum. This is because, when the temperature increase above the optimum, the decomposition reactions of cellular compounds, such as proteins and nucleic acids, are largely favoured, and at the same time, irreversible damage, takes place in the plasmatic membrane, causing dispersion of the cellular components, abruptly reducing the metabolic functions (12). In the literature, E_a and E_b values for different types of microorganisms have been put forward (13, 14).

However, the optimum temperature for *T. ferrooxidans* has not been precisely defined because it is subject to variation between different strains. The optimum temperature has been recognized to be pH dependent, in that a decrease in pH lowers the optimum temperature for growth and iron oxidation (15, 16). For example, the optimum temperature of *T. ferrooxidans* is 33°C at pH 2.5 and 30°C at pH 1.5 (16). The upper temperature limit is approximately 42 to 43°C (17), whereas the lower temperature range is not well defined for these bacteria. Iron oxidation has been demonstrated to occur at temperatures as low as 5 to 6°C (18, 19).

MATERIALS AND METHODS

Microorganism The strain of *T. ferrooxidans* used in this study was isolated in the Riotinto mines of Huelva (Spain) and kindly made available by the Biohydrometallurgy Group of the University of Seville (Spain). Later, this strain was purified by several subcultures on solid media.

Medium and cultivation conditions The medium used to grow and maintain the microorganism was that proposed by Silverman *et al.* (20) in g/*l*: $(NH_4)_2SO_4$ 3.0; $MgSO_4$ 0.5; K_2HPO_4 0.5; KCl 0.1; $Ca(NO_3)_2$ 0.01 and a concentration of $FeSO_4$ of 2.0 g/*l*. Erlenmeyer flasks of 500 ml were used, containing 200 ml medium and 10% of inoculum. Independent experimental runs were conducted, at different temperatures between 15°C and 40°C, at different pH values between 2.3 and 1.25 and at agitation rates between zero and 250 rpm.

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Solid medium The solid medium for subcultures and viable biomass determination was that proposed by Johnson *et al.* (21), known as FeTSBo. This medium was prepared as follows: the three separately sterilized solutions (TSB/basal salts, ferrous sulphate and agarose) were combined, thoroughly mixed and the complete medium divided 2 : 1 by volume and held at 50°C. To the larger volume was added 2.5% (v/v) of an active culture of heterotrophic acidophile, the solution was again thoroughly mixed and 20 ml aliquots dispensed onto sterile Petri plates. When the agarose had gelled, it was covered with a thin layer (10 ml) of sterile FeTSB (which had been kept molten at 50°C).

Analytical methods The oxidation of ferrous sulphate was monitored by determining its residual concentration in the medium following the 1,10-phenantroline method (22). In order to measure the concentration of total iron in solution the ferric iron was reduced to ferrous, after filtration of the medium, using hydroxylamine as reducing agent and determining this concentration by the previously mentioned method. Subsequently, the concentration of ferric iron in solution was determined by difference between the ferrous and total iron concentration.

The concentration of total biomass was determined by counting in a Neubauer chamber with optical microscope following the classical method. Viable biomass determination was made by plate counting. Each measurement was made in duplicate to reduce the experimental errors inherent in working with microbial populations.

RESULTS AND DISCUSSION

Influence of temperature Experimental data of ferrous iron (g/*l*), ferric iron (g/*l*) and total biomass (Mcells/ml) versus time (h), for each temperature studied, are showed in Figs. 1 to 6. It is necessary to point out that the region of greatest interest in our study was the exponential growth phase, because in this phase, cells grow at a constant specific growth rate, for constant medium conditions and an accurate estimation of μ_c is possible.

Maximum specific growth rate data (μ_{max}) were calculated taking into account specific growth rate (μ_c), calculated from knowledge at the concentrations of ferrous and ferric iron and the kinetic equation proposed by Gómez *et al.* (10):

$$\mu_c = \frac{\mu_{max} \cdot Fe^{2+}}{K_S + Fe^{2+} + K_I \cdot Fe^{3+}}$$

In this equation $K_S = 0.94$ g/*l* and $K_I = 2.98$, as can be found in Gómez *et al.* (10). The study of the values obtained for μ_{max} suggested that a Sinclair's type equation was adequate for its behaviour:

$$\mu_{max} = A \cdot e^{-E_a/RT} - B \cdot e^{-E_b/RT}$$

In order to adjust the experimental data (μ_{max}) obtained to this typical equation, a non linear regression method based on the Marquardt algorithm (23) was used. At low temperature, the cellular multiplication term prevails, while at temperatures above 30–40°C, the thermal deactivation term dominates. In a qualitative way, the starting iteration values of E_a and E_b can be evaluated from the average slopes of experimental maximum specific growth rate data. Having carried out the required calculations using the Marquardt algorithm, the

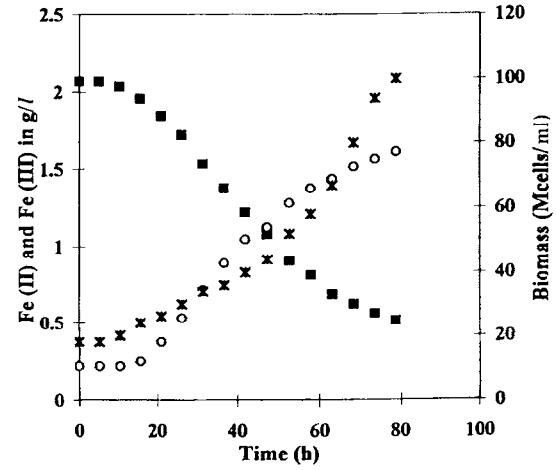

FIG. 2. Experimental data of ferrous iron, ferric iron and biomass versus time at 20°C. Symbols: ■, Fe (II); ○, Fe (III); ●, biomass.

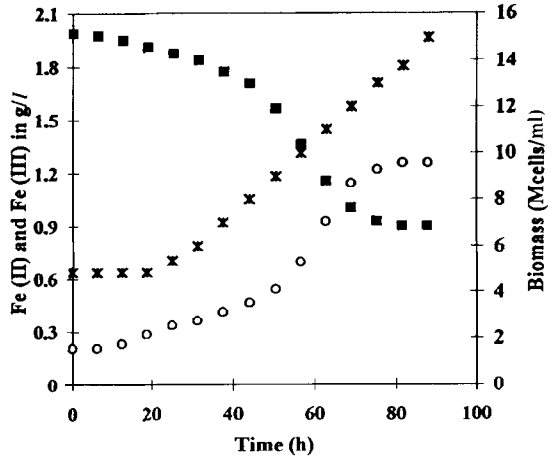

FIG. 1. Experimental data of ferrous iron, ferric iron and biomass versus time at 15°C. Symbols: ■, Fe (II); ○, Fe (III); ●, biomass.

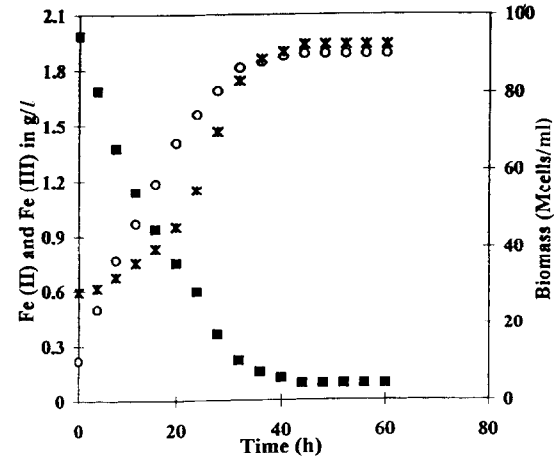

FIG. 3. Experimental data of ferrous iron, ferric iron and biomass versus time at 25°C. Symbols: ■, Fe (II); ○, Fe (III); ●, biomass.

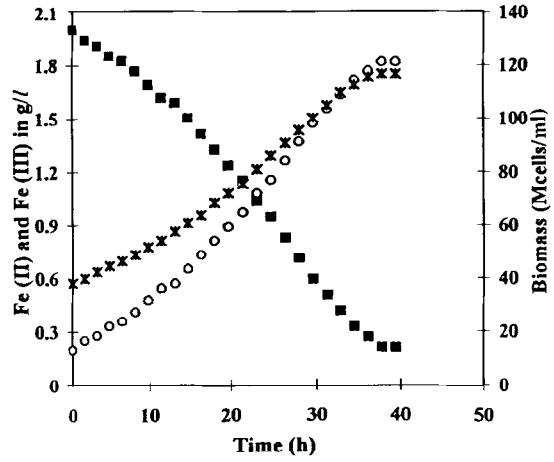

FIG. 4. Experimental data of ferrous iron, ferric iron and biomass versus time at 30°C. Symbols: ■, Fe (II); ○, Fe (III); ●, biomass.

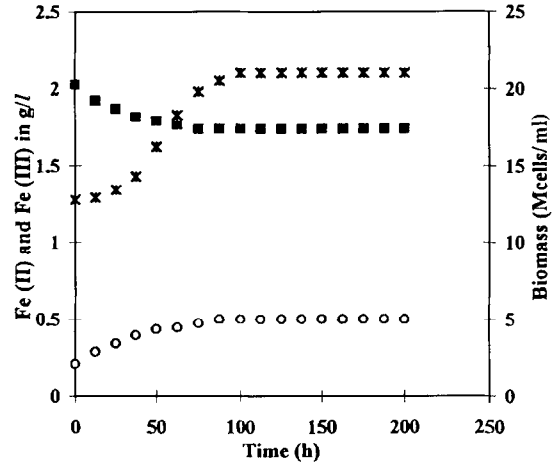

FIG. 6. Experimental data of ferrous iron, ferric iron and biomass versus time at 40°C. Symbols: ■, Fe (II); ○, Fe (III); ●, biomass.

following values of the temperature equation parameters were obtained: $A=2.50\,h^{-1}$; $E_a=164.92$ cal/mol; $B=3.1\cdot10^3\,h^{-1}$; $E_b=705.56$ cal/mol and $r^2=0.95$ with $R=1.987$ cal/°C·mol.

In Fig. 7, the theoretical predictions and experimental data of maximum specific growth rates (h^{-1}) for *T. ferrooxidans* versus temperature (°C) can be observed. The representation of the maximum specific growth rate of *T. ferrooxidans* versus the operating temperature is of a skewed Gauss type. At temperatures above the optimum, bacterial deactivation processes are correlated with the denaturation of essential enzymes; the scattering of membrane cellular constituents and an increase of the sensitivity to the toxic effect of ferric iron are observed. Moreover, the energy required for these processes is greater than that used for the bacterial activation, which explains why the optimum temperature is closer to the maximum of μ_{max} curve than to the minimum.

The temperature below which it is considered that there is no growth of *T. ferrooxidans* (less than 1% of the optimum maximum specific growth rate) appears to be about 11°C. The maximum temperature value from which the microorganisms growth is totally inhibited is around 40°C. Finally, according to the equation obtained, the optimum temperature for the maximum specific growth rate is 31°C.

The activation energy values calculated may be compared with those proposed in the literature for other microorganisms (13, 14). For a particular type of yeast, energies associated with a multiplication factor of 10^4 cal/mol were obtained, appearing clearly superior to those calculate for *T. ferrooxidans*. Further, the denaturation activation energies are also higher in yeasts (about 10^5 cal/mol); suggesting that, in general, above the optimum, *T. ferrooxidans* suffer the harmful effects of temperature less than yeast do. At the same time, E_a values are similar to those proposed for acetic acid bacteria; but, in constrat, E_b are higher for this type of bacteria. This fact suggests that *T. ferrooxidans* is more resistant to high temperature levels than *Acetobacter aceti*.

In short, the proposed equation for μ_{max} behaviour and the values of the involved energies (E_a and E_b) can be useful for the election of a working temperature in ferrous sulphate oxidation processes, making possible

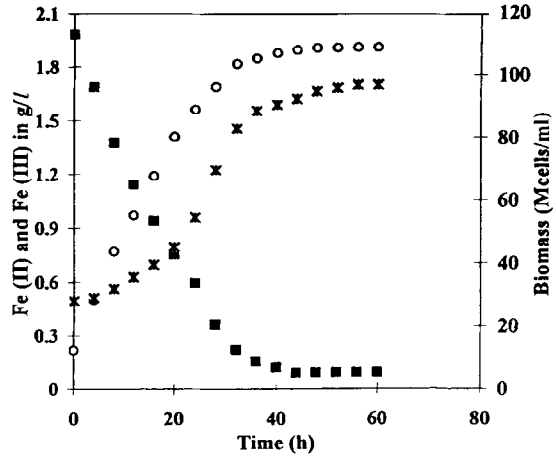

FIG. 5. Experimental data of ferrous iron, ferric iron and biomass versus time at 35°C. Symbols: ■, Fe (II); ○, Fe (III); ●, biomass.

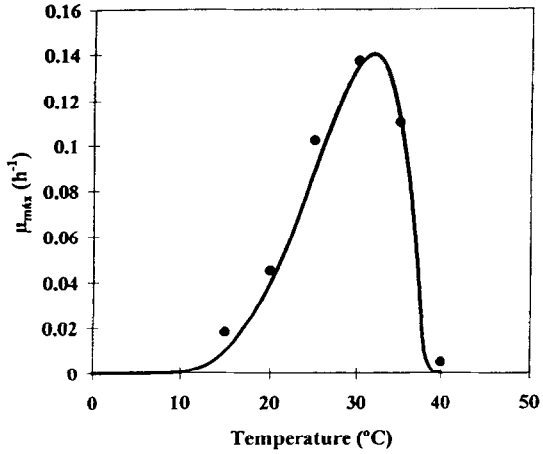

FIG. 7. Theoretical predictions and experimental data of maximum specific growth rate (h^{-1}) for *T. ferrooxidans* versus temperature. Symbols: ●, experimental; —, theoretical.

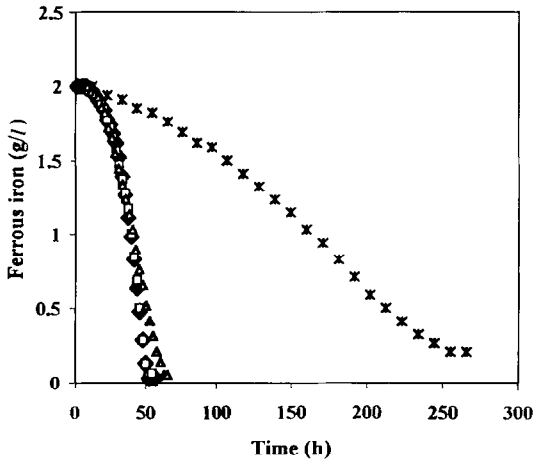

FIG. 8. Experimental data of ferrous iron versus time at different pH values. Symbols: ◆, pH 2.5; □, pH 2.0; △, pH 1.5; ●, pH 1.25.

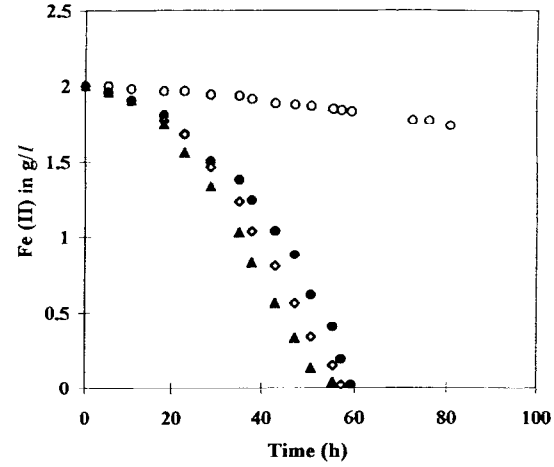

FIG. 10. Experimental data of ferrous iron versus time at different agitation rates. Symbols: ○, 0 rpm; ●, 100 rpm; ◇, 200 rpm; ▲, 250 rpm.

the operation under optimum conditions.

Influence of initial pH value Figure 8 gives the results of the determinations of Fe (II) for the different pH values tested. At first sight, it is observed that there no differences between the experiment conducted at pH of 2.5, 2.0 and 1.5 with respect to consumption of substrate. However, it should be pointed out that pH values below 1.8 are effective in limiting the formation of precipitates associated with the microbial metabolism (5, 24). These precipitates have a series of negative effects on bioreactors in which oxidation of ferrous sulphate is produced; among the more important of these is the formation of physical barriers as a result of the low diffusion of the reagents and products throughout the precipitation zone; futhermore, they can block the pumps, valves, pipework and other auxiliary equipment (25).

In addition, the results of the determinations of precipitate quantities formed at the end of each experiment show a sharp reduction as the pH is reduced; by reducing the initial pH from 2.5 to 2.0, a reduction of up to 63% can be obtained in these precipitates.

Similarly, the pH value influences the viability of the cellular population. To demonstrate this, cultures of *T. ferrooxidans* were inoculated at two pH values, 2.0 and 1.25. When all the substrate had been used up, counting the colonies gave the results shown in Fig. 9. As can be observed, *T. ferrooxidans* presents a sharp reduction in viability when working at a pH of 1.25, with the population decreasing by a factor of 1000 after 7 d. However, this effect is not so marked when working at an initial pH of 2.0.

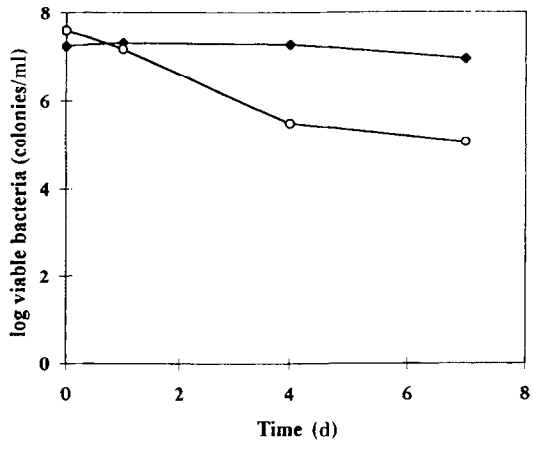

FIG. 9. Effect of pH value on cell viability. Symbols: ●, pH 2.0; ○, pH 1.25.

The pH value of this medium was determined during the experiments and a rising trend was observed, with this increase (in all cases less than four tenths of a point) in pH being sharper in those cultures with a lower initial pH. This tendency is a direct consequence of the main reaction of oxidation of Fe (II) to Fe (III). Therefore, it can be stated that it would be useful to buffer the cultures of *T. ferrooxidans* to an initial pH of 1.8, so that the series of reactions taking place during the oxidation process leave the final pH value at around 2.0 to 2.1. At this value, the kinetics of the biological reaction reach an optimum value and, in addition, ensure that the formation of insoluble compounds of Fe (III) is considerably reduced.

Influence of the rate of agitation The experimental data are reported in Fig. 10, in which the concentration of Fe (II) against time is shown for the different rates of agitation tested.

The results show that there are no significant differences in respect of substrate consumption when working in the range between 100 and 250 rpm. This finding confirms that the demand for dissolved oxygen by *T. ferrooxidans* is very low. In contrast, there are appreciable differences compared with the experiment conducted without agitation; here it can be observed that the transport of oxygen across the gas-liquid interface is not sufficient and a growth takes place in conditions of limiting oxygen.

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