Appl Microbiol Biotechnol (1999) 51: 813–819

ORIGINAL PAPER

M. Boon · C. Ras · J. J. Heijnen

The ferrous iron oxidation kinetics of *Thiobacillus ferrooxidans* in batch cultures

Received: 8 February 1999 / Accepted: 17 February 1999

Abstract The ferrous iron oxidation kinetics of *Thiobacillus ferrooxidans* in batch cultures was examined, using on-line off-gas analyses to measure the oxygen and carbon dioxide consumption rates continuously. A cell suspension from continuous cultures at steady state was used as the inoculum. It was observed that a dynamic phase occurred in the initial phase of the experiment. In this phase the bacterial ferrous iron oxidation and growth were uncoupled. After about 16 h the bacteria were adapted and achieved a pseudo-steady state, in which the specific growth rate and oxygen consumption rate were coupled and their relationship was described by the Pirt equation. In pseudo-steady state, the growth and oxidation kinetics were accurately described by the rate equation for competitive product inhibition. Bacterial substrate consumption is regarded as the primary process, which is described by the equation for competitive product inhibition. Subsequently the kinetic equation for the specific growth rate, μ, is derived by applying the Pirt equation for bacterial substrate consumption and growth. The maximum specific growth rate, μ_{max}, measured in the batch culture agrees with the dilution rate at which washout occurs in continuous cultures. The maximum oxygen consumption rate, $q_{O_2,max}$, of the cell suspension in the batch culture was determined by respiration measurements in a biological oxygen monitor at excess ferrous iron, and showed changes of up to 20% during the course of the experiment. The kinetic constants determined in the batch culture slightly differ from those in continuous cultures, such that, at equal ferric to ferrous iron concentration ratios, biomass-specific rates are up to 1.3 times higher in continuous cultures.

M. Boon (✉) · C. Ras · J. J. Heijnen
Department of Biochemical Engineering, Kluyver Laboratory of Biotechnology, Julianalaan 67, 2628 BC Delft, The Netherlands
e-mail: M.Boon@stm.tudelft.nl
Tel.: +31-15-2781551
Fax: +31-15-2782355

Introduction

Several authors have determined the bacterial ferrous iron oxidation kinetics of *Thiobacillus ferrooxidans,* using different experimental systems (continuous cultures, batch cultures, and initial rate measurements were reviewed in Boon 1996). Because kinetic data derived from different experimental systems were not compared or mutually related, it is not clear whether the kinetics is independent of the measurement method. In a batch culture, adaptation of bacteria to the new conditions might be significant and will only occur after one or more doubling times. In the last phase of a batch culture experiment, bacteria grow at a rapidly decreasing ferrous iron concentration, which again is non-steady state. A pseudo-steady state occurs if the bacteria do not adapt their internal system to the changing external conditions and the steady-state kinetics applies. In order to discover whether adaptation of *T. ferrooxidans* to changing conditions has a significant effect on the ferrous iron oxidation kinetics, the work described in this paper focused on the kinetics in batch culture experiments.

The measurement technique used in our work differs in several aspects from batch culture experiments reported so far (Lacey and Lawson 1970; Kelly and Jones 1978; Braddock et al. 1984; Lui et al. 1988; Shrihari et al. 1990). (i) Inocula for the cultures are taken from continuous cultures at steady state, which ensures that the conditions in which the bacteria are grown are accurately known. (ii) On-line off-gas analyses of oxygen and carbon dioxide are applied, which ensures that the oxygen and carbon dioxide consumption rate, the biomass-specific ferrous iron and oxygen consumption rates and the specific growth rate, as well as the biomass and ferrous iron concentrations, are continuously known (Boon et al. 1998a). (iii) The maximum biomass-specific oxygen consumption rates, $q_{O_2,max}$, of a cell suspension in the batch culture were determined several times during the course of a batch culture experiment, using a biological oxygen monitor (Boon 1996).

Theory

The bacterial ferrous iron oxidation and oxygen consumption are coupled according to the degree of reduction balance for autotrophic bacterial growth on ferrous iron (Boon et al. 1995a, 1998a). In deriving this equation it was assumed that bacteria have the composition $CH_{1.8}O_{0.5}N_{0.2}$ (Roels 1983):

$$-r_{Fe^{2+}} = -4r_{O_2} - 4.2r_{CO_2} \quad (1)$$

where $-r_{Fe^{2+}}$ is the ferrous iron oxidation rate (mol Fe^{2+} l^{-1} s^{-1}), $-r_{O_2}$ is the O_2 consumption rate (mol O_2 l slurry^{-1} s^{-1}) and $-r_{CO_2}$ is the CO_2 consumption rate (mol CO_2 l slurry^{-1} s^{-1}).

The energy generated from the bacterial oxidation of ferrous iron is used by the bacteria for both growth rate and maintenance. This is described in the Pirt equation (Pirt 1982):

$$-r_{Fe^{2+}} = \frac{r_x}{Y_{sx}^{max}} + m_s \cdot c_x \quad (2)$$

where r_x is the bacterial growth rate (C-mol l^{-1} s^{-1}), Y_{sx}^{max} is the maximum yield coefficient of biomass on ferrous iron (C-mol/mol Fe^{2+}), m_s is the maintenance coefficient of biomass on ferrous iron (mol Fe^{2+} C-mol^{-1} s^{-1}) and c_x is the biomass concentration (C-mol/l). This can be rewritten as a relationship between the biomass-specific oxygen consumption rate (q_{O_2}, mol O_2 C-mol^{-1} s^{-1}) and the specific growth rate, μ (s^{-1}):

$$q_{O_2} = \frac{\mu}{Y_{ox}^{max}} + m_o \quad (3)$$

The values of Y_{ox}^{max} [the maximum yield coefficient on oxygen (C-mol/mol O_2] and m_o [the maintenance coefficient on O_2 (mol O_2 C-mol^{-1} s^{-1})] are derived from the measurements using the following equation:

$$\frac{1}{Y_{ox}} = \frac{1}{Y_{ox}^{max}} + \frac{m_o}{\mu} \quad (4)$$

In this equation Y_{ox} is the yield of biomass on oxygen (C-mol/mol O_2), which is directly calculated from the oxygen and carbon dioxide consumption rate: $Y_{ox} = r_{CO_2}/r_{O_2}$ Thus, plotting $1/Y_{ox}$ against $1/\mu$, yields m_o as the slope and $1/Y_{ox}^{max}$ as the intercept.

If the degree of reduction balance (Eq. 1) applies, the maximum yield coefficients for iron, Y_{sx}^{max}, and oxygen, Y_{ox}^{max}, are related and only one of these two parameters needs to be determined:

$$Y_{ox}^{max} = \frac{4Y_{sx}^{max}}{1 - 4.2Y_{sx}^{max}} \quad (5)$$

The same holds for the maintenance coefficients, m_o and m_s:

$$m_o = \frac{m_s}{4} \quad (6)$$

If the Pirt equation applies, the biomass-specific growth rate is directly related to the specific oxygen consumption rate (Eq. 3), which mathematically relates the specific growth kinetics to the kinetic equation for the specific oxygen consumption. Assuming that the maximum specific growth and oxygen consumption rate coincide, the following relation between μ_{max} and $q_{O_2,max}$ holds:

$$q_{O_2,max} = \frac{\mu_{max}}{Y_{ox}^{max}} + m_o \quad (7)$$

In our work it is assumed that substrate and oxygen consumption are the primary processes. It has been shown that the bacterial oxidation rate of ferrous iron is strictly stoichiometrically related to the oxygen consumption rate under all circumstances (Boon et al. 1995a). Accordingly, the ferrous oxidation rate is calculated from the oxygen consumption rate by applying the stoichiometric equation for bacterial growth on ferrous iron (Eq. 1 in Boon et al. 1998a). In this work on batch culture experiments it has been found that a threshold level of ferrous iron occurs at which the oxygen consumption is terminated. Therefore, the Monod equation for competitive ferric iron inhibition has been adapted for the occurrence of a threshold ferrous iron concentration, $[Fe^{2+}]_t$ (mol/l):

$$q_{O_2} = \frac{q_{O_2,max}}{1 + \frac{K_s}{[Fe^{2+}] - [Fe^{2+}]_t} + \frac{K_s}{K_i} \cdot \frac{[Fe^{3+}]}{[Fe^{2+}] - [Fe^{2+}]_t}} \quad (8)$$

where K_s is the Monod constant for ferrous iron (mol Fe^{2+}/l) K_i is the inhibition constant (mol Fe^{3+}/l).

If the Pirt equation applies, the growth kinetics is easily determined from the kinetics of oxygen or substrate consumption. Using Eqs. 8, 3 and 7, an equation for the specific growth kinetics is derived:

$$\mu = \frac{\mu_{max} + m_o \cdot Y_{ox}^{max}}{1 + \frac{K_s}{[Fe^{2+}] - [Fe^{2+}]_t} + \frac{K_s}{K_i} \cdot \frac{[Fe^{3+}]}{[Fe^{2+}] - [Fe^{2+}]_t}} - m_o \cdot Y_{ox}^{max} \quad (9)$$

This equation takes into account the fact that, at concentrations near the threshold ferrous iron concentration, $[Fe^{2+}]_t$, a negative specific growth rate will occur (i.e. bacterial decay), which is due to maintenance requirements of the bacteria, while oxygen and ferrous iron are still consumed under these conditions. It has been shown in previous work (Boon 1996) that, at the relatively high concentrations of total iron in batch culture measurements, the value of the denominator is insensitive to the value of the second term, $K_s/([Fe^{2+} - Fe^{2+}]_t)$, because the value of K_s is very low (K_s = 0.2 mM). Consequently, only the value of K_s/K_i and not the separate values of K_s and K_i are accurately determined from batch culture experiments.

Materials and methods

Batch culture experiments were carried out in a stirred and aerated fermenter with on-line off-gas analyses. At the start of the experiment, a cell suspension from a continuous culture was added as an inoculum (5% or 10% v/v) to a solution of ferrous iron in medium.

The effect of the initial growth rate of the inoculum was examined by inoculating with cell suspensions from continuous cultures at different dilution rates (thus $\mu_{inoculum} = D$, the dilution rate of the continuous culture, $l\ l^{-1}\ h^{-1}$). The use of the off-gas analyses in the batch culture for determination of the biomass and ferrous iron concentration and the biomass-specific rates has been explained in a previous paper (see Figs 4–8 in Boon et al. 1995a).

In order to describe the bacterial zinc sulfide oxidation kinetics with *T. ferrooxidans*, the ferrous iron oxidation kinetics in the presence of zinc ions needs to be known (Boon et al 1995b, 1998b). Therefore, several batch culture experiments with additional zinc ions ($ZnSO_4 \cdot 7H_2O$, J.T. Baker, Holland) were carried out. The analytical method to determine total iron and ferrous iron concentrations (the colorimetric *ortho*-phenanthroline method, according to ASTM D1068) needs to be adapted if the sample contains zinc ions (Boon 1996). In Table 1 the experimental conditions are given at which batch culture experiments were carried out.

A description of the equipment, analytical methods, characteristics of the bacteria, media and chemicals has been given elsewhere (Boon et al. 1999). On-line off-gas analyses of O_2 and CO_2 in the off-gas and reference air occurred every 20 min. In the initial phase of the batch culture experiment the oxygen consumption rate in undiluted cell suspension was also measured by the biological oxygen monitor (BOM) (Boon 1996). This provided the opportunity to compare the oxygen consumption rate measured in the BOM (with a Clark cell) and r_{O_2} (mol $l^{-1}\ h^{-1}$) determined from O_2 analyses in the off-gas. In order to determine the oxygen consumption rate more frequently in the final phase of the experiment, BOM measurements with undiluted samples were also carried out every 5–10 min in this phase. Stationary BOM measurements were also used as a tool to determine the maximum oxygen consumption rate, $q_{O_2,max}$, of the cell suspension during the batch culture measurement. The ferrous and ferric iron concentrations in the batch culture were determined in samples every 30 min, and every 5 min in the final phase. pH control in these measurements was by hand (every hour) by the addition of 1 M H_2SO_4.

Results

Conversion rates and concentrations in batch culture experiments

In all batch culture experiments it was found that, within the accuracy of the analytical procedures, the measured total organic carbon was equal to the total amount of carbon consumed (as carbon dioxide), which indicates that the carbon balance has been met and the biomass concentration during the course of the experiment is accurately determined from the on-line CO_2 analyses in the gas phases (cf. Fig. 5 in Boon et al. 1995a). It has also been found that the ferrous iron concentration calculated from the degree of reduction balance (Eq. 1) coincides with $[Fe^{2+}]$ determined in *ortho*-phenanthroline analyses (cf. Fig. 6 in Boon et al. 1995a). Therefore, the degree of reduction balance applies and the ferrous iron concentration in a batch culture at time t is accurately calculated from the oxygen and carbon dioxide consumption (Eq. 1).

It was found in the experiments that a threshold level of ferrous iron, $[Fe^{2+}]_t$, occurred, at which oxygen consumption terminated. The measured values of $[Fe^{2+}]_t$ are listed in Table 1. Accordingly, the Monod equation for competitive ferric iron inhibition, which has been used in previous papers (Eqs. 5 and 8 in Boon

Table 1 Batch culture experiments on ferrous iron with inoculum cell suspensions from continuous cultures with *Thiobacillus ferrooxidans* and different steady states, $D_{inoculum}$, and 0.21 M Fe, pH 1.8, 30 °C. In the column "Measured values", μ_{max} and $q_{O_2,max}$ are maximum values determined in the batch culture from off-gas analyses; $q_{O_2,max}$ (biological O_2 monitor, *BOM*) shows the range of values of $q_{O_2,max}$ in batch samples measured in the BOM in the last 20 h (pseudo-steady state) of the batch culture experiment and $[Fe^{2+}]_t$ is the measured concentration of ferrous iron in the batch at which oxygen consumption is terminated. In the column "Kinetic constants" μ_{max}, $q_{O_2,max}$ and K_s/K_i are values that are fitted for the last 20 h (pseudo-steady state) of the batch culture measurement. In continuous culture $D_{washout} = 0.096\ h^{-1}$, and the fitted kinetic parameters for continuous cultures were $q_{O_2,max} = 2.2$ molO_2 mol$CO_2^{-1}\ h^{-1}$ and $K_s/K_i = 0.05$

$D_{inoculum}$ (h^{-1})	Inoculum (% v/v)	$[Fe]_{total}$ (mol/l)	Remarks	Measured values				Kinetic constants		
				μ_{max} measured after ±16 h (h^{-1})	$q_{O_2,max}$ measured in initial 16 h (mol C-mol$^{-1}\ h^{-1}$)	$q_{O_2,max}$ BOM last 20 h (mol C-mol$^{-1}\ h^{-1}$)	$[Fe^{2+}]_t$ (mM)	μ_{max} fitted (h^{-1})	$q_{O_2,max}$ fitted (mol C-mol$^{-1}\ h^{-1}$)	K_s/K_i fitted
0.014	10	0.21	Duplicate	0.093	2.2 ± 0.1	1.8–2.1	0.45	0.095	2.4	0.10 ± 0.005
0.033	10	0.21	Duplicate	0.102	2.4 ± 0.1	2.0–2.35	0.52	0.105	2.3	0.06 ± 0.005
0.040	5 and 10	0.21	One each	0.094	2.3 ± 0.1	1.8–2.2	0.55	0.100	2.2	0.06 ± 0.005
0.085	10	0.21	Duplicate	0.085	2.2 ± 0.1	1.5–1.7		0.085	1.7	0.10 ± 0.005
0.042	10	0.27						0.06 ± 0.01	1.2 ± 0.1	0.06 ± 0.01
0.042	10	0.36						0.065 ± 0.005	1.2 ± 0.1	0.055 ± 0.005
0.042	10	0.21	0.15 M $ZnSO_4$					0.06 ± 0.01	1.2 ± 0.1	0.06 ± 0.01
0.042	10	0.21	0.31 M $ZnSO_4$					0.05 ± 0.01	1.0 ± 0.1	0.06 ± 0.01

et al. 1995b) has been adapted, which yielded Eq. 8 and Eq. 9, used in this paper.

Kinetics at 0.21 M total iron

Figure 1 shows a typical plot of the biomass-specific growth rate ($\mu = r_{CO_2}/c_x$), against the ferric to ferrous iron concentration ratio in a batch culture experiment. Also μ, calculated with Eq. 9 (with $Y_{ox}^{max} m_o = 0.006\ h^{-1}$) is plotted. The fitted kinetic constants, μ_{max} and K_s/K_i, for different batch culture experiments are listed in Table 1. In the initial phase of the batch culture experiment ($t < 16$ h and $[Fe^{3+}]/[Fe^{2+}] < 0.6$) the measured value of μ increases and a deviation between its calculated and measured values occurs. After 12–16 h the ferric to ferrous ratio is between 0.4 and 0.6, and a maximum value of the specific growth rate is measured. The measured values of μ_{max} in different batch culture experiments are given in Table 1. In all batch culture experiments the fitted value of μ_{max} was equal to the observed maximum specific growth rate (see Table 1).

Figure 2 shows a typical plot of the biomass-specific oxygen consumption rate ($q_{O_2} = r_{O_2}/c_x$) against the ferric to ferrous iron concentration ratio in a batch culture experiment. In contrast to the specific growth rate, the biomass-specific oxygen consumption rate reaches a high value immediately the inoculum is added to the batch. The biomass-specific oxygen consumption rate has also been calculated from the equation for competitive inhibition kinetics (Eq. 8) and plotted in this graph. The fitted kinetic constants, $q_{O_2,max}$ and K_s/K_i, for different batch culture experiments are listed in Table 1.

The maximum oxygen consumption rate in batch culture samples was also frequently measured in the BOM. In Boon et al. (1995a) Fig. 8, an example of the values of $q_{O_2,max}$ measured in the BOM during the batch culture experiment was presented. The value of $q_{O_2,max}$ that is directly determined from the on-line analyses of oxygen in the off-gas agrees with the value of $q_{O_2,max}$ determined in the BOM. Table 1 lists the average value of $q_{O_2,max}$ in the initial 16 h of the batch culture experiment, determined both from the off-gas analyses at $[Fe^{3+}]/[Fe^{2+}]$ between 0.1 and 0.2 and from BOM measurements (these values coincide). The average value of $q_{O_2,max}$ determined in frequent BOM measurements during the last 20 h ($[Fe^{3+}]/[Fe^{2+}] > 0.6$) are also listed in Table 1. From these data it can be seen that a decrease of 10%–20% in the value of $q_{O_2,max}$ occurs after the initial 16 h in the batch culture.

In the pseudo-steady-state phase the same values of K_s/K_i (see Table 1) were obtained for the kinetic description of μ and q_{O_2}, and also μ_{max} and $q_{O_2,max}$ are related according to Eq. 7. This result is in accordance with the applicability of the Pirt equation in the pseudo-steady state (see below).

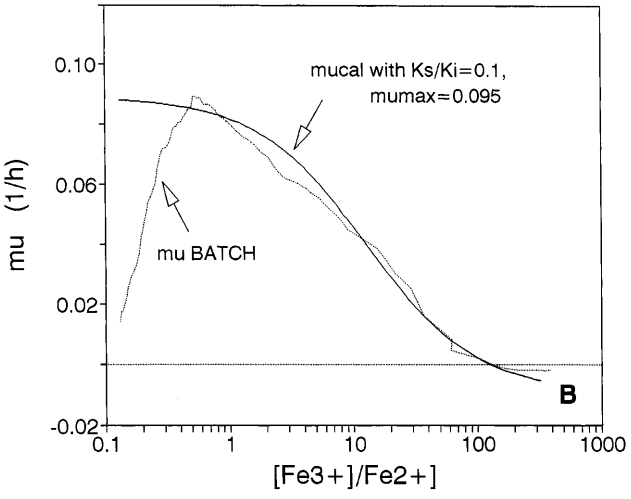

Fig. 1 Measured biomass-specific growth rate (···) in batch culture at 0.21 M total iron (same batch culture as shown in Boon et al. 1995a). Inoculum: 0.21 mol Fe/l, 10% inoculum, $D_{inoculum} = 0.014\ h^{-1}$. — μ calculated with kinetic equation and given parameters

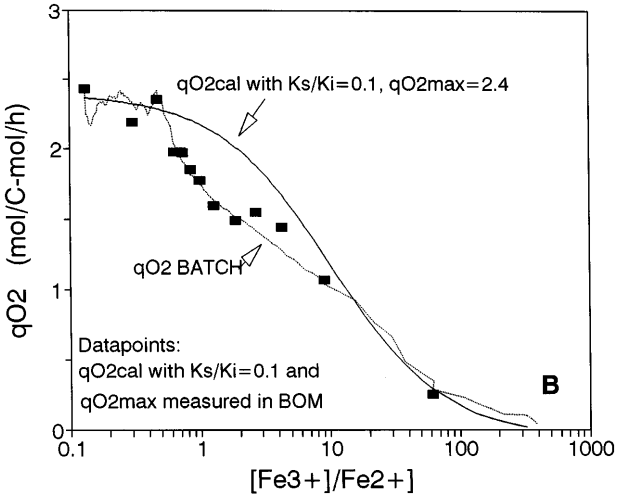

Fig. 2 Measured biomass-specific oxygen consumption rate (...): 0.21 mol Fe/l, 10% inoculum, $D_{inoculum} = 0.014\ h^{-1}$. — q_{O_2} calculated with kinetic equation and a fitted value of $q_{O_2,max}$. ■ q_{O_2} calculated with the same equation using the values of $q_{O_2,max}$ measured in the biological O_2 monitor

Yield and maintenance coefficients in batch cultures

While the ferrous iron oxidation (derived from the chemical analyses) and oxygen consumption were coupled according to the degree of reduction balance during the whole batch experiment, the maximum yield coefficients for iron, Y_{sx}^{max}, and oxygen, Y_{ox}^{max}, are also related according to the degree of reduction balance. Therefore, only one of these two parameters needs to be determined (Eq. 5). The same holds for the maintenance coefficients, m_s and m_o (Eq. 6). The Pirt equation is applied to describe the pseudo-steady-state phase (last 20 h) in

the batch culture experiment (see Fig. 3), which yields $Y_{ox}^{max} = 0.052 + 0.002$ C-mol/mol O_2 and $m_o = 0.24 +$ 50% mol O_2/C-mol/h.

Kinetics at other conditions

In order to examine the effect of the total iron concentration on the kinetics, batch culture experiments were carried out at 0.27 M and 0.36 M total iron. *T. ferrooxidans* grown in continuous culture were used as the inoculum (0.21 M total iron, $D = 0.04\ h^{-1}$). At the start of the experiment the specific oxygen consumption rate was equal to the value of $q_{O_2,max}$ at 0.21 M ferrous iron, but then slowly decreased within 30 h to a new constant level. In this whole phase, sufficient ferrous iron was available for $q_{O_2,max}$ to occur (i.e. $[Fe^{3+}]/[Fe^{2+}] < 0.1$). In this phase the specific growth rate initially increased similarly to μ in batch cultures at 0.21 M total iron, but subsequently decreased. A pseudo-steady-state was observed in the last 20 h of the experiments, which could be described with the equation for competitive inhibition kinetics and using the value of K_s/K_i equal to that for cultures at 0.21 M total iron but lower values of μ_{max} and $q_{O_2,max}$ (Table 1). The values of $q_{O_2,max}$ and μ_{max} at 0.27 M total iron are not significantly different from the values at 0.36 M total iron. It is concluded that higher total iron concentrations cause a decrease of the maximum specific oxygen consumption and growth rates, whereas the kinetic parameter K_s/K_i remains equal to that at 0.21 M total iron.

The inhibiting effect of zinc ions on the bacterial ferrous iron oxidation was examined in two batch culture experiments with 0.21 M ferrous iron solution to which 0.15 and 0.30 M of zinc sulfate was added. Cells grown in continuous culture were used as the inoculum (0.21 M total iron, $D = 0.04\ h^{-1}$). The behaviour in the initial 30 h of the experiment was similar to that in the batch culture at high iron concentration. Again, the last 20 h of the experiment were accurately described by the equation for competitive inhibition kinetics and using the value of K_s/K_i equal to that for cultures at 0.21 M total iron but lower values of μ_{max} and $q_{O_2,max}$ (Table 1). The values of $q_{O_2,max}$ and μ_{max} at 0.15 M added zinc ions are not significantly different from the values at 0.31 M zinc. It is concluded that the addition of zinc ions causes a decrease of the maximum specific oxygen consumption and growth rates, whereas the kinetic parameter K_s/K_i remains constant. Batch cultures at 0.21 M ferrous iron were completely deactivated within 5 h after the addition of 0.8 M zinc sulfate.

Discussion

It was expected that the maximum specific growth rate of the bacteria, μ_{max}, in a continuous culture could be easily measured in batch culture experiments according to the same principle as that governing the measurement of $q_{O_2,max}$ of these cells in BOM measurements at high ferrous iron, which is the independent measurement of the carbon dioxide consumption rate from the on-line off-gas analyses at a high ferrous concentration in the initial phase of the batch culture experiment. According to the kinetic equation (Eq. 9), at low ratios of $[Fe^{3+}]/[Fe^{2+}]$ (i.e. high ferrous iron) in the initial phase of the batch culture experiment, μ_{max} would occur (see solid curve in Fig. 1 at $[Fe^{3+}]/[Fe^{2+}] < 0.6$). However, the measured specific growth rate increased in the initial 16 h of the batch culture experiments and only after 12–16 h ($[Fe^{3+}]/[Fe^{2+}]$ between 0.4 and 0.6) was a maximum value measured. Figure 4 shows the specific

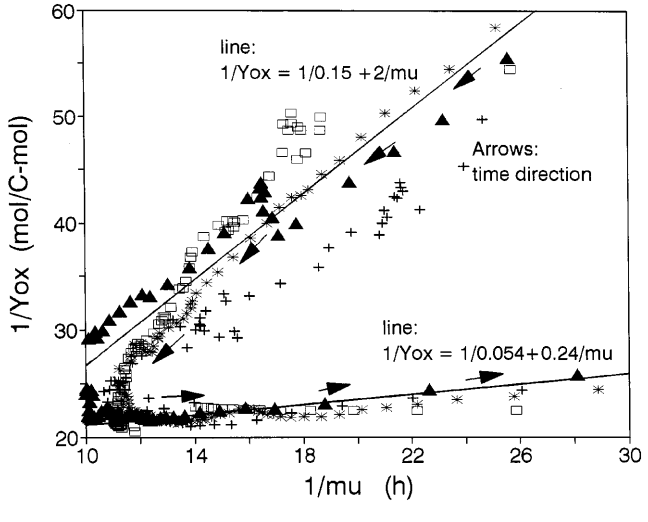

Fig. 3 Comparison of reciprocal yield of *Thiobacillus ferrooxidans* ($=r_{O_2}/r_{CO_2}$) in the initial phase of batch cultures at 0.21 M Fe * $D_{inoculum} = 0.014$, ▲ $D_{inoculum} = 0.033$, + $D_{inoculum} = 0.033$, □ $D_{inoculum} = 0.040\ h^{-1}$

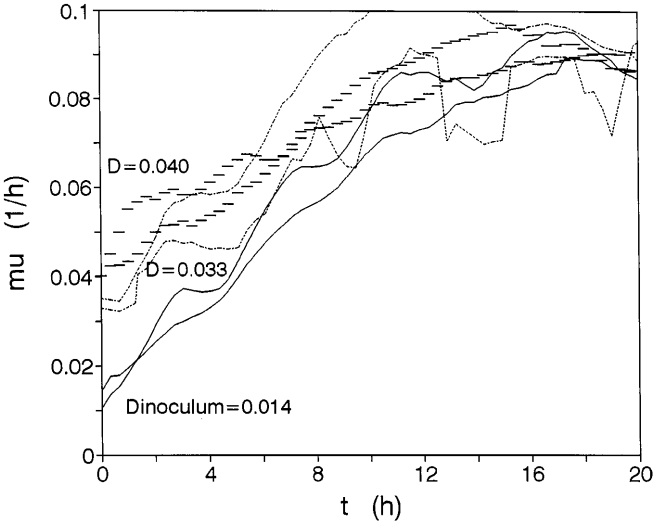

Fig. 4 Biomass-specific growth rate in the initial 20 h of six batch cultures at 0.21 M Fe. — $D_{inoculum} = 0.014$, ... $D_{inoculum} = 0.033$, - - - $D_{inoculum} = 0.033$

growth rate in the initial 20 h of six batch culture experiments. Inocula at three different dilution rates were used. It was found that the specific growth rate at $t = 0$ is very close to the specific growth rate of the inoculum ($D_{inoculum}$). In all batch culture experiments 16 ± 2 h was needed from the start of the experiment to achieve $\mu_{max} \approx 0.1\ h^{-1}$. No correlation was observed between the time needed to achieve the maximum specific growth rate and the value of $D_{inoculum}$. Apparently the acceleration of the specific growth rate was larger when lower $D_{inoculum}$ was used. It is concluded that, in contrast to the specific oxygen consumption rate of cells grown in continuous culture, the specific growth rate needs to adapt when the cells are transferred from low to high ferrous iron concentration, and the specific growth rate in this dynamic phase cannot be described by competitive inhibition kinetics (see Fig. 1 and compare with Fig. 2).

The use of the on-line off-gas analyses allows that the bacterial growth rate, μ, and oxygen consumption rate to be measured very accurately. Only from these measurements does it become obvious that the specific growth rate shows dynamic behaviour when the cells are transferred from a low ferrous iron concentration in the continuous culture to a high ferrous iron concentration in the batch culture. Apparently *T. ferrooxidans* needs to adapt when transferred from a low (continuous culture) to a high (batch culture) ferrous iron concentration. It is proposed that major changes in the anabolic system can occur, which cause dynamic behaviour of the growth kinetics, whereas only minor changes of the catabolic system occur under changing external conditions. Consequently, bacterial growth and substrate consumption are uncoupled when bacteria are transferred from low to high ferrous iron.

The initial growth rate of the inoculum did not significantly affect the measured value of μ_{max} (compare μ_{max} at varying $D_{inoculum}$ in Table 1). Only at $D_{inoculum} = 0.085\ h^{-1}$ was a slightly lower value of μ_{max} observed. In order to test whether no further increase of the measured specific growth rate would occur if excess ferrous iron was available for a longer period, an inoculum size of only 5% was used in one of the experiments. In this experiment the measured maximum growth rate remained constant at $\mu_{max} = 0.093 \pm 0.003$ for about 4 h, which implies that the maximum specific growth rate was achieved at this level. The average value of μ_{max} in the batch culture agrees with $D_{washout}$ in continuous cultures (Boon et al. 1999).

After the dynamic phase (after 16 h) a pseudo-steady state (the last 20 h of the experiment) was observed in which the measured value of μ agreed with the value calculated with Eq. 9. It is concluded that competitive inhibition kinetics (Eq. 9) gives a good description of the measured specific growth rate in the batch culture only in the pseudo-steady-state phase of the batch culture experiment. In Fig. 1 it can be seen that at $[Fe^{3+}]/[Fe^{2+}] > 100$ the measured biomass-specific growth rate was negative (i.e. CO_2 was produced), which is also predicted by the model. This decay of bacteria is due to the fact that the consumption rate of substrate (which is still larger than zero, see Fig. 2) is insufficient to provide for the maintenance requirements of the bacteria.

While the specific growth rate that is observed after 12–16 h agrees with the fitted value of μ_{max} in Eq. 9 (see Table 1), and because the measured average value of μ_{max} is close to $D_{washout}$ in continuous cultures, batch culture experiments with on-line off-gas analyses are useful to determine the value of μ_{max} of *T. ferrooxidans* on ferrous iron.

The growth kinetics in the pseudo-steady-state phase of the batch culture experiment is close to that in continuous cultures (Boon et al. 1999). However, because $K_s/K_i = 0.04 \pm 0.01$ in continuous cultures is lower than the value of 0.08 ± 0.02 in batch cultures, at high ratios of $[Fe^{3+}]/[Fe^{2+}]$ the specific growth rate in continuous culture is up to 1.3 times higher (see Fig. 9 in Boon et al. 1999). Thus, at equal specific growth rates (μ in batch culture is equal to D in continuous culture), higher values of $[Fe^{3+}]/[Fe^{2+}]$ are obtained in continuous cultures.

A significant decrease of $q_{O_2,max}$ measured in BOM experiments was observed in all batch culture experiments as soon as the bacteria had achieved their maximum growth rate (after about 16 h, see Table 1). Apparently the maximum oxygen consumption rate of the bacteria decreases at high specific growth rates. This behaviour agrees with the reproducible decrease of $q_{O_2,max}$ (measured in the BOM) in continuous cultures at high dilution rates (Boon et al. 1999). Also, in Fig. 2 it can be seen that a deviation occurs between the value of q_{O_2} calculated from the kinetic model (solid line) and the measured value of q_{O_2} (dotted line) at $[Fe^{3+}]/[Fe^{2+}]$ ratios between 0.5 and 10. Now, by calculating q_{O_2} and using the measured (BOM) values of $q_{O_2,max}$ in the kinetic equation instead of a fitted (constant) value of $q_{O_2,max}$, a much better description of q_{O_2} in the batch culture values is obtained (see data points in Fig. 2). Therefore, it is concluded that the value of $q_{O_2,max}$ is not a constant value but is related to the specific growth rate of the bacteria. BOM measurements at high ferrous iron concentration are a tool to determine the actual value of $q_{O_2,max}$ in a cell suspension.

Dynamic behaviour is also observed at higher total iron concentrations or when zinc ions are added to the batch culture. A pseudo-steady state is obtained after about 30 h which is appropriately described by the kinetic equation. Under these conditions, the values of $q_{O_2,max}$ and μ_{max} are 40%–50% lower while the kinetic parameter K_s/K_i remains constant. However, it is not clear whether batch culture measurements are appropriate to predict the kinetic effects of pH, total iron or toxic ions at steady state. In continuous cultures at higher total iron (0.27 M), no such strong decrease of $q_{O_2,max}$ was observed. This implies that batch culture experiments are not appropriate to examine the effect of the total iron concentration on the steady-state kinetics. It is not clear whether the measured inhibition of zinc

ions describes cells that have adapted in a continuous culture that contains zinc ions. Most likely, adaptation of the bacteria to new or toxic conditions requires more than two or three bacterial doubling times. To examine the kinetics in batch cultures would require much smaller inoculum sizes to be used. It is concluded that batch culture experiments are inappropriate for examining the effect of pH, total iron concentration and toxic ions on the steady-state behaviour of *T. ferrooxidans*. These effects are only properly examined in continuous culture.

In the pseudo-steady state the value of $Y_{ox}^{max} = 0.052 \pm 0.001$ C-mol/mol O_2 agrees with that in continuous cultures ($Y_{ox}^{max} = 0.051 \pm 0.001$ C-mol/mol O_2, Boon et al. 1999). The maintenance coefficient in the pseudo-steady state, m_o = 0.24 + 50% mol O_2 C-mol^{-1} h^{-1} is about double that in continuous cultures (m_o = 0.1 ± 0.02 mol O_2 C-mol^{-1} h^{-1}). Because Y_{ox}^{max} and m_o determined in the batch culture are less reliable (their values largely depend on which data points are used in the linear regression) it is concluded that the energetic parameters in continuous cultures and in the pseudo-steady state of batch cultures do not significantly differ.

In Figure 3 the reciprocal yield, $1/Y_{ox} (= r_{O_2}/r_{CO_2})$, of *T. ferrooxidans* in six batch cultures is plotted against the reciprocal specific growth rate, $1/\mu$. This figure shows that the Pirt equation does not apply because two different yields are achieved at one growth rate. This effect is caused by the dynamic behaviour in the initial phase of the batch culture experiments (arrows give the time course in this plot). The Pirt equation was also used to describe the yield on oxygen in the dynamic phase of the six experiments, giving $Y_{ox}^{max} = 0.15$ C-mol/mol O_2 and m_o = 2 mol O_2 C-mol^{-1} h^{-1}. Whether these values have a physical meaning is not clear. Apparently the actual yield of biomass on oxygen, Y_{ox}, in the dynamic phase (initial 16 h) is very low. This indicates that much less energy is available for bacterial growth when bacteria adapt to the high ferrous iron concentration. It is important to notice that the initial growth rate ($D_{inoculum}$) does not significantly affect the yield on oxygen, Y_{ox}, as a function of the growth rate, μ, in this dynamic phase, which indicates that the energy use of bacteria in their adaptation phase is not affected by their initial conditions.

Summarizing, the equation for competitive product inhibition with a threshold ferrous iron concentration describes the growth and oxidation kinetics of the pseudo-steady state in batch cultures. In these phases the growth and oxidation kinetics are coupled according to the Pirt equation. A slightly better kinetic description of q_{O_2} is obtained when the values of $q_{O_2,max}$ measured in the BOM are used.

Acknowledgements D. Reuvers and J.P. Lispet contributed significantly to the experimental part of this work.

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