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Determination of Kinetic and Stoichiometric Parameters of *Pseudomonas putida* F1 by Chemostat and *In Situ* Pulse Respirometry

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Determination of Kinetic and Stoichiometric Parameters of *Pseudomonas putida* F1 by Chemostat and *In Situ* Pulse Respirometry*

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Abstract

The applicability of pulse respirometry, for the estimation of kinetic and stoichiometric parameters in pure cultures was evaluated by comparison with traditional chemostat method. *Pseudomonas putida* F1 was cultured in a continuous stirred tank reactor, using glucose as sole carbon source. The reactor was operated under steady-state with six dilution rates, ranging from 0.06 to 0.35 h^{-1}. Substrate and biomass concentration were measured and used to estimate kinetic and stoichiometric parameters, according to the Monod model. An *in situ* respirometry method was also applied to the reactor, with the injection of pulses of glucose from 19 to 97 mg L^{-1}. The respirograms obtained were used to estimate the kinetic and stoichiometric parameters according to ASM1 and ASM3 models. No significance difference was observed between parameters estimated by chemostat and respirometric methods. The glucose affinity constant was from 0.4 to 0.7 mg L^{-1}, the maximum specific growth rate was from varying from 0.14 to 0.20 h^{-1}, and the growth yield was from 0.41 to 0.67. These results confirm that *in situ* pulse respirometry is a suitable method for kinetic and stoichiometric parameters estimation.

KEYWORDS: affinity constant, growth rate, ASM1, ASM3, Monod

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INTRODUCTION

Development and optimization of new bioprocesses requires continuous information about the kinetic and stoichiometric parameters of the microorganisms used in those processes (Sipkema et al., 1998). The estimation of these parameters for pure cultures is traditionally performed through batch or chemostat culture. Among these two, the chemostat is considered the most suitable method for determining substrate affinity constant (K_S, Duarte *et al.*, 1994). It consists in measuring the residual limiting substrate concentration for different dilution rates applied to a chemostat. This continuous culture, just as the batch method, involves the direct measurement of the growth limiting substrate at concentrations close to the K_S (Kovarova-Kovar and Egli, 1998). Koch (1997) pointed out the precision and accuracy of substrate measurements as the main limitation of the method. Additionally, traditional chemostat method is time consuming, as it requires the operation of the reactor under several steady-states, each of them maintained for several hydraulic retention times (*HRT*).

Drawbacks of traditional methods may be overcome by respirometry, which allows the indirect measurement of substrate consumption rates by monitoring the biological oxygen consumption rate, under well defined conditions (Spanjer *et al.*, 1999). Within many respirometry techniques, pulse respirometry, developed in the 1990s (Riefler *et al.,* 1998; Vanrolleghem *et al.*, 1995; Kong *et al*., 1994) is probably one of the most promising techniques. This technique consists of measuring the dissolved oxygen (*DO*) concentration during the transient state observed after the injection of a defined concentration of substrate into the system. The exogenous oxygen uptake rate (*OUR*) curves reflect the kinetic of the aerobic biodegradation process. The interest of respirometry for parameter estimation, compared with techniques based on substrate concentration measurement, is that respirometry allows the retrieval of numerous parameters from relatively small experimental effort in real time, and using a low cost probe (Riefler *et al.*, 1998). Additionally, since pulse respirometry is based on the observation of induced transient states, it may be applied without changing the normal operation of the system.

Many works on respirometry can be found in literature, with a special emphasis given to parameters retrievability, precision, and sensitivity analysis. Comparatively, little weight has been given to the accuracy of the retrieved parameters. Their comparison to parameters retrieved by more standard methods is often avoided. Pulse respirometry has been often used to characterize mixed cultures applied to wastewater treatment, such as nitrificants (Ordaz *et al.*, 2008) and activated sludge (Kong *et al.*, 1994). There are few works that used respirometric techniques to characterize pure cultures. Goudar and Strevett (1998) used *OUR* measurements for the estimation of growth kinetic parameters of

Penicillium chrysogenum. Latter, Insel *et al.* (2006) used respirometry and glucose consumption profiles to obtain kinetic parameters of *Escherichia coli*. In this last mentioned work, process kinetic was described by a mathematic model similar to Activated Sludge Model 3 (*ASM3*). However, little attention was given to the accuracy of the retrieved parameters.

In the present work, pulse respirometry was applied for the retrieval of kinetic and stoichiometric parameters of a pure culture of a *Pseudomonas putida* F1 strain fed with glucose as sole carbon source in a continuous stirred reactor. *In situ* pulse respirometry, defined as pulses of substrate directly applied to biological reactors as reported previously by Ordaz *et al.* (2008), was used. The *ASM1* (Henze *et al.*, 1987) and the *ASM3* (Gujer *et al.*, 1999) models were used for respirometric data interpretation.

To assess the accuracy of the proposed method, the kinetic and stoichiometric parameters retrieved from respirometric experiments were compared to those obtained through traditional chemostat method.

MATERIAL AND METHODS

BIOREACTOR

A continuous stirred tank reactor (*CSTR*, Bioflo3000, New Brunswick Scientific, Mexico) with a working volume of 3 L was used. Air was continuously supplied at an air flow rate of 5 L min^{-1}. The reactor was continuously fed at defined flow rates. The composition of the growth media used was (g L^{-1}): KH_2PO_4, 1.25; $NH_4(SO_4)_2$, 1.32; $MgSO_4.7H_2O$, 0.10; $FeSO_4 \cdot 7H_2O$, 0.75. Additionally, 1 mL L^{-1} of trace elements (Vecht *et al.*, 1988) was added, as well as 5 g L^{-1} of glucose as sole carbon source. Temperature and pH in the reactor were maintained constant at 25ºC and 7, respectively.

MICROORGANISM AND CULTURE CONDITIONS

The microorganism used was *Pseudomonas putida* F1 (CDBB – B100, National Collection of Microbial Strains, Cinvestav, Mexico). The preparation of the inoculum was made in a 2 L Erlenmeyer flask containing 600 mL of culture medium, incubated for 24 h at 30 ºC and 200 rpm in an orbital shaker (Innova4300, New Brunswick Scientific, Mexico).

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CHEMOSTAT METHOD

For the estimation of kinetic and stoichiometric parameters through chemostat method, the bioreactor was operated continuously at six dilution rates (*D*): 0.06, 0.09, 0.10, 0.17, 0.21 and 0.35 h^{-1}. Each *D* was maintained until steady-state was reached and kept for at least 6 *HRT* (from 30 to 100 h). Steady-state was considered to be reached when cellular and residual substrate concentrations were stable, within 10 % variation.

PULSE RESPIROMETRY METHOD

The respirometric methodology was previously reported by Ordaz *et al.* (2008). This procedure is shortly remembered here: (i) the *DO* data acquisition was started; (ii) the substrate feeding of the reactor was then stopped; (iii) after the *DO* concentration reached a pseudo steady-state, called baseline (C_b), a known concentration of substrate (S_P), namely from 19 to 97 mg L^{-1} was injected in the reactor; (iv) the *DO* concentration was monitored until it reached the base line again; finally (v) the mass transfer coefficient (k_La) of the reactor was measured in triplicate. Respirograms were obtained by plotting the *DO* concentration *versus* time. All the pulse respirometric experiments were made during the operation of the reactor at a single dilution rate (D = 0.17 h^{-1}), because pulse respirometry is based on the observation of induced transient states in the reactor operated temporarily under batch mode and therefore independent of the dilution rate.

ANALYTICAL METHODS

Influent and effluent were characterized by the measurement of glucose, and total and soluble chemical oxygen demand (*COD*). Glucose concentration was measured by two methods depending on the concentration; an enzymatic kit (Amplex Red, Invitrogen, USA) for the 1 to 100 mg L^{-1} range and *DNS* (dinitrosalicylic acid) method for concentrations over 100 mg L^{-1}. *COD* was measured by the closed reflux colorimetric method, in accordance to Standard Methods (APHA, 1999). Total *COD* was determined by measuring the *COD* of a homogenized sample. Soluble *COD* (*S*) was determined by measuring the *COD* of a filtered sample (0.45 μm). Insoluble *COD* was estimated as the difference between total and soluble *COD*. Since the feeding medium contained no suspended solids, the insoluble *COD* was considered as biomass (*X*). *DO* concentration was measured continuously in the reactor with a polarographic probe (InPro6800, Mettler Toledo, México). The *DO* probe was connected to a computer for data acquisition. The calibration of the *DO* probe was done using water at 25 ºC, bubbled with nitrogen gas for 0 % and with air for 100 %

saturation. The k_La was measured in triplicate using the dynamic method as described by Badino *et al.* (2000).

DATA ANALYSIS

CHEMOSTAT METHOD

The determination of the maximum specific growth rate (μ_{max}) and K_S by the chemostat method was made by adjustment of the Monod equation to the experimental data by least-square fit. Since the bioreactor used was a *CSTR*, the specific growth rate (μ) was considered equal to D. The biomass growth yield ($Y_{X/S}$) was determined through *COD* mass balance.

PULSE RESPIROMETRIC METHOD

The oxygen mass balance during pulse experiments can be described by Equation 1 (Kong *et al.*, 1994) in which the response time (*tr*) of the process and of the *DO* electrode are taken into account as described previously by Vanrolleghem *et al.* (2004). In Equation 1, the exogenous oxygen uptake rate (OUR_{ex}) can be described by a simple Monod kinetic (*ASM1*) or a more complex model (*ASM3,* Table 1). ASM1 is an unstructured model for the simulation of oxygen and substrate consumption associated to biomass growth. *ASM3* is a partially structured model for the simulation of oxygen and substrate consumption associated to storage and growth mechanisms. The choice of these two models was based on their simplicity and their wide application in the field of respirometry.

$$\frac{dC}{dt} = \left(k_L a(C_b - C) - OUR_{ex}\right)\left(1 - e^{-\frac{t}{tr}}\right) \qquad (1)$$

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Table 1. Simplified matrix of *ASM1* and *ASM3* for organic carbon removal, considering soluble biodegradable *COD*

Component **Process**	***S***	X_{Sto}	***X***	***C***	**Rate**
Growth on S (*ASM1*)	$-1/Y_{X/S}$		1	$-(1-Y_{X/S})/Y_{X/S}$	$\mu_{max} \cdot X \cdot \frac{S}{S+K_S}$
Storage of S (*ASM3*)	-1	$Y_{Sto/S}$		$-(1-Y_{Sto/S})/Y_{Sto/S}$	$k_{Sto} \cdot X \cdot \frac{S}{S+K'_S}$
Growth on X_{Sto} (*ASM3*)		$-1/Y'_{X/S}$	1	$-(1-Y'_{X/S})/Y'_{X/S}$	$\mu_{max} \cdot X \cdot \frac{X_{Sto}/X}{X_{Sto}/X + K_{Sto}}$

ASM1 PARAMETERS ESTIMATION

The oxidation yield ($Y_{O_2/S}$) was first retrieved from the area of the respirograms according to Equation 2 (Ordaz *et al*., 2008). When a known amount of substrate is oxidized during the pulse injection, the $Y_{O_2/S}$ is given by the amount of oxygen consumed per unit *COD* of substrate oxidized (S_p). As both biomass and substrate are expressed in *COD* units, $Y_{X/S}$ can be easily estimated from $Y_{O_2/S}$ (Equation 3).

$$Y_{O_2/S} = \frac{\int_0^t OUR_{ex}\,dt}{S_P} = \frac{k_L a \int_0^t (C_b - C)dt + (C_0 - C_f)}{S_P} \tag{2}$$

$$Y_{X/S} = 1 - Y_{O_2/S} = 1 - \frac{k_L a \int_0^t (C_b - C)dt + (C_0 - C_f)}{S_P} \tag{3}$$

In Equation 2 and 3, S_p is the substrate pulse concentration, and C_0 and C_f are the *DO* concentrations before and after the pulse, respectively. The accumulation term ($C_0 - C_f$) can be omitted, as no difference between C_0 and C_f was observed during pulse experiments. K_S and *tr* were estimated by adjustment of *ASM1* to the experimental data obtained from the pulse experiments with a fitting procedure based on Runge-Kutta method and a Marquardt optimization method with 20 convergence steps (Model Maker, Cherwell Scientific Publishing, UK). μ_{max} was estimated from Equation 4, where, $OUR_{ex}max$ is the maximum

OUR_{ex}, and it was obtained according to Ordaz *et al.* (2008) method, *i.e.* by the injection of substrate pulse of increasing concentration.

$$\mu_{max} = \frac{OUR_{ex}\, max \cdot Y_{X/S}}{Y_{O_2/S} \cdot X} \tag{4}$$

ASM3 PARAMETERS ESTIMATION

As pointed out above, *ASM3* model considers storage. Storage and growth are considered sequentially: first, all the substrate is consumed for storage; and then bacteria consume storage material for growth. Two growth yields are therefore defined: storage yield ($Y_{Sto/S}$), and storage growth yield ($Y_{X/Sto}$). Both parameters were estimated according to Equations 5 and 6 (Karahan-Gül *et al.*, 2002; Goel *et al.*, 1999).

$$Y_{Sto/S} = 1 - \frac{k_L a \int_0^t (C_{LSto} - C)dt}{S_P} \tag{5}$$

$$Y_{X/Sto} = \frac{Y_{X/S}}{Y_{Sto/S}} \tag{6}$$

Some of the kinetic and stoichiometric coefficients were set to default values. These default values were those reported by Karahan-Gül *et al.* (2002), and are listed in Table 2. The additional kinetic and stoichiometric parameters of the *ASM3* model, namely the soluble substrate affinity (K'_S), the storage affinity constant (K_{Sto}), the storage kinetic constant (k_{Sto}), and *tr* were estimated by adjustment of *ASM3* to the experimental data obtained from the pulse experiments with a fitting procedure based on Runge-Kutta method and a Marquardt optimization method with 20 convergence steps (Model Maker, Cherwell Scientific Publishing, UK). μ_{max} was estimated from Equation 4.

Table 2. Kinetic and stoichiometric coefficients used in the ASM3 model (Karahan-Gül *et al.* 2002)

Model coefficient	**Default Values (h^{-1})**
b_h	0.0083
b_{Sto}	0.0083

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STATISTICAL ANALYSIS OF THE RESULTS

The parameters obtained by respirometry and chemostat methods were compared using the Tuckey-Kramer tests performed after analysis of variance (α = 0.05) using the NCSS 2000 software (NCSS, Jerry Hintze). The impact of the number of measurements on the standard error (S_E) was estimated from Equation 7 (Freund and Wilson, 1996), were n was considered as the number of independent pulse experiments in respirometry and the number of steady-states in the chemostat method.

$$S_E = \frac{\sigma}{\sqrt{n}} \tag{7}$$

RESULTS AND DISCUSSION

During a 12 days experiment, the *CSTR* was operated under steady-state with six successive D (0.06, 0.09, 0.10, 0.17, 0.21, and 0.35 h^{-1}). At D = 0.35 h^{-1}, a clear biomass wash-out was observed. Thus, the results obtained at D 0.35 h^{-1} were discarded. Figure 1 presents the adjustment of the Monod equation to the experimental data obtained with the chemostat method, which had a correlation factor (r^2) of 0.955. Average kinetic and stoichiometric parameters are presented in Table 3.

By the end of the chemostat experiment, the reactor was maintained under steady-state at D = 0.17 h^{-1}, and the pulse respirometry experiments were made. The reactor was maintained until stable residual glucose concentration was observed, and a total of 9 pulses were injected in a row, during a non-stop 36 hours experiment. Figure 2 shows an example of respirogram observed after the injection of a pulse of 97 mg L^{-1}. The *DO* concentration descended sharply from 7.4 to 5.0 mg L^{-1}, followed by a stable period, and then by a sharp increase up to the initial steady-state. Similar curve shapes were observed in all respirometric experiments (results not shown). *ASM1*, based on Monod kinetic, did not fit adequately the obtained respirograms (average r^2 = 0.957). On the contrary, *ASM3* model did (average r^2 = 0.985). This is clearly shown in Figure 2 were *ASM1* and *ASM3* best fittings are presented. The existence of storage and storage material degradation are therefore suggested. This is also suggested from the shape of respirograms. Indeed, the stable *DO* concentration observed in the middle of the pulses (*i.e.* from 0.05 to 0.15 h in Figure 2) as well as the sudden change in the *OUR* slope followed by tailing (Figure 2), point to the existence of storage mechanisms (Guisasola *et al*., 2005, Van Loosdrecht *et al.*, 1997).

Table 3 shows the average kinetic and stoichiometric parameter values estimated by the chemostat method, and respirometry by *ASM1* and *ASM3* model

adjustment to the respirometric data. This Table confirms that *ASM3* model better fits the experimental respirometric data and allows the retrieval of a major number of stoichiometric and kinetic parameters. K_S of *Pseudomonas putida* F1 strain was ranging from 4.86 to 9.30 mg L^{-1}. This range of K_S is significantly inferior to values reported in literature for *Pseudomonas putida* (20 to 1000 mg L^{-1}, Wang *et al*., 1996; Vicente and Cánovas, 1973). The $Y_{X/S}$ of *Pseudomonas putida* F1 obtained by direct measurement in the chemostat was 0.41. This value is lower than 0.51 to 0.67 estimated by respirometry, which indicates that respirometry probably overestimates the $Y_{X/S}$. The μ_{max} was varying from 0.14 to 0.20 h^{-1}. As observed during chemostat experiment, the reactor was operated successfully at D = 0.21 h^{-1}, without significant wash-out. The μ_{max} was therefore probably underestimated by both chemostat and respirometry methods.

Table 3. Kinetic and stoichiometric parameters with standard error. Letters in parenthesis are Tukey-Kramer (*TK*) test results (α = 0.05). Values with the same capital letter are not significantly different

Parameter	Chemostat	Respirometry (*ASM1*)	Respirometry (*ASM3*)
$Y_{X/S}$ (D = 0.17 h^{-1})	0.41 ± 0.05 (A)	0.51 ± 0.04 (A)	-
$Y'_{X/S}$ (D = 0.17 h^{-1})	-	-	0.67 ± 0.03
$Y_{Sto/S}$	-	-	0.79 ± 0.08
K_S (mg L^{-1})	4.86 ± 0.70 (B)	7.96 ± 2.65 (B)	-
K'_S (mg L^{-1})	-	-	5.13 ± 1.99
K_{Sto} (mg L^{-1})	-	-	0.01 ± 0.003
μ_{max} (h^{-1})	0.20 ± 0.05 (C)	0.14 ± 0.02 (C)	0.16 ± 0.01 (C)
k_{Sto} (h^{-1})	-	-	0.28 ± 0.07
tr (s)	-	40.46 ± 4.34	20.85 ± 3.28
r^2	0.955	0.957 ± 0.005	0.985 ± 0.004

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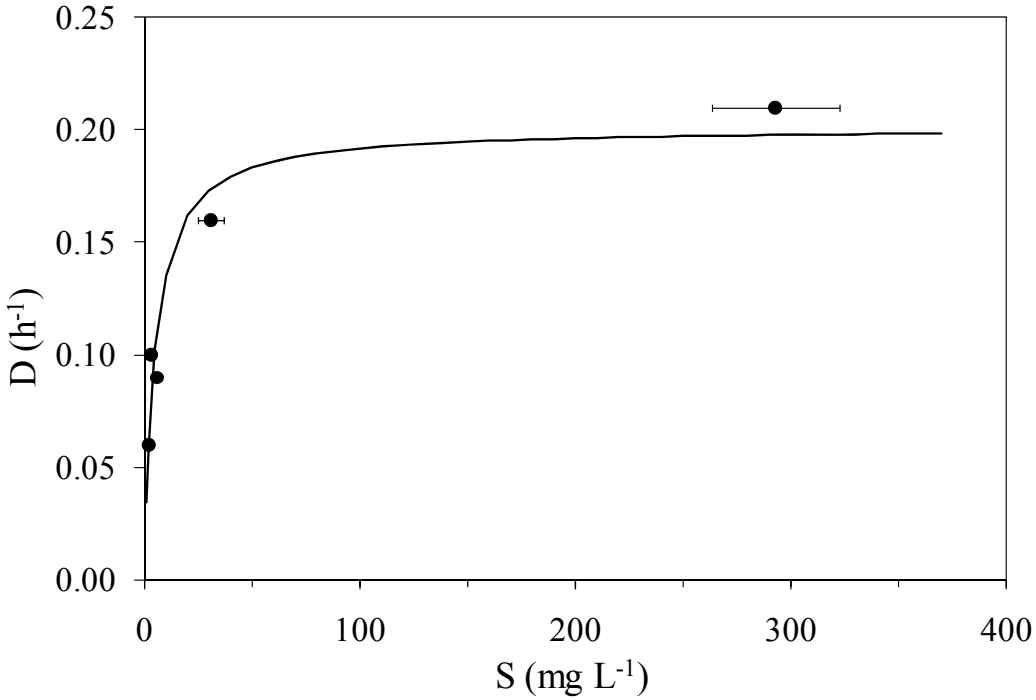

Figure 1. Monod model adjustment (r^2 = 0.955, solid line) to the experimental chemostat data (○).

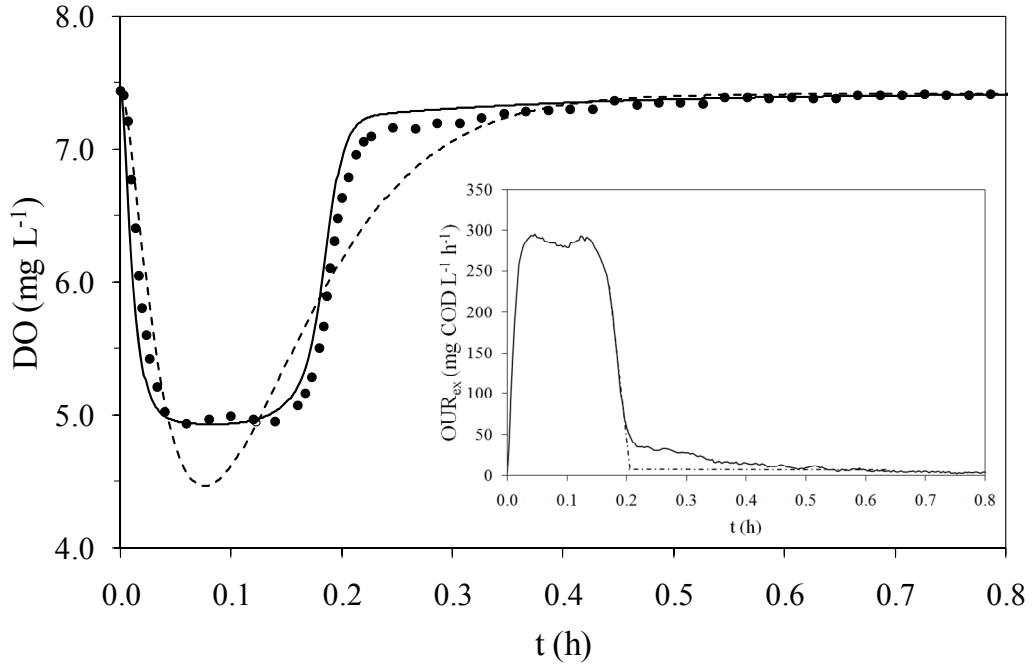

Figure 2. Example a respirogram (•), and model fitting using *ASM1* (r^2 = 0.935, dotted line) and *ASM3* (r^2 = 0.997, solid line). Figure within the main frame: corresponding *OUR* curve (solid line) and *ASM1*- typical fitting (dash-dotted line).

A special attention should be given to the accuracy and precision of parameter estimation. *ASM3* includes several parameters that are not considered in Monod or *ASM1* models. Kinetic and stoichiometric parameters obtained from *ASM3* model cannot be compared to others, except μ_{max}. On the contrary, *ASM1* and Monod are similar and can be compared. Table 3 shows the results of the Tuckey-Kramer comparison tests. No difference between *ASM1*-respirometry, and chemostat methods were observed for any of the parameters. In the same way, no significant difference between μ_{max} estimated by *ASM1* and *ASM3* models was observed.

Table 4 shows the S_E of parameters estimation, for n = 5, 10, and 20. $Y_{X/S}$ and μ_{max} were subject to lower S_E when measured by respirometry than by chemostat. On the other hand, the chemostat method allowed the estimation of the K_S with a lower S_E than the respirometric method, for the same number of replicate. The number of replicates of pulse respirometry for a given S_E is about four times the required number of steady-states in the chemostat method. However, pulse experiments are much less time demanding than chemostat methods, which requires several steady-states. Each steady-state has to be maintained for several *HRT*, which represented in this work from 17 to 100 h of experiment. By comparison, pulse experiments lasted from 0.4 to 0.6 h. It can be therefore concluded that pulse respirometry is a technique that allows similar K_S estimation with a relatively lower experimental effort. The same conclusion can be withdrawn for μ_{max} and $Y_{X/S}$ estimation, since S_E of respirometry estimation was lower than chemostat estimation.

Table 4. Standard error of kinetic and stoichiometric parameters

Parameter	Method	*n*		
		5	10	20
$Y_{X/S}$	*ASM1*	10.5 %	7.4 %	5.3 %
	Chemostat	12.0 %	8.5 %	6.0 %
$Y'_{X/S}$	*ASM3*	5.3 %	3.7 %	2.6 %
K_S	*ASM1*	44.7 %	31.6 %	22.3 %
	Chemostat	14.4 %	10.2 %	7.2 %
K'_S	*ASM3*	52.0 %	36.7 %	26.0 %
K_{Sto}	*ASM3*	44.7 %	31.6 %	22.4 %
k_{Sto}	*ASM3*	33.5 %	23.7 %	16.8 %
μ_{max}	*ASM1*	16.0 %	11.3 %	8.0 %
	Chemostat	24.6 %	17.4 %	12.3 %
	ASM3	5.6 %	4.0 %	2.8 %

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CONCLUSIONS

From the results obtained, it can be confirmed that respirometry is a suitable method for parameter estimation of axenic culture, as previously suggested by Goudar and Strevett (1998) and by Insel et al. (2006). The *ASM3* model fitted adequately the respirometry kinetic of *Pseudomonos putida* F1 with an average r^2 of 0.985. This confirms that *Pseudomonas putida* F1 metabolism includes storage mechanisms. This was previously reported by Huijberts and co-workers (1992), and explains why *ASM1* model showed a lower correlation coefficient (average r^2 of 0.957) than *ASM3*. A comparison, between the parameters estimated by respirometry and chemostat methods showed that no significant difference was observed for any parameter. This is partly due to the relatively large S_E observed in parameter estimation. Respirometry method showed lower S_E than chemostat method for $Y_{X/S}$ and μ_{max} estimation, but larger S_E for K_S estimation. However, as respirometry implies relatively lower experimental input, it can be concluded that *in situ* pulse respirometry is an effective method for parameter estimation of axenic cultures of *Pseudomonas putida* F1.

NOMENCLATURE

α	Type I error
μ	Specific growth rate (h^{-1})
μ_{max}	Maximum specific growth rate (h^{-1})
σ	Standard deviation
ASM1; 3	Activated sludge model 1; and 3
b_h	ASM3 endogenous respiration rate of biomass (h^{-1})
b_{Sto}	ASM3 endogenous respiration rate of storage material (h^{-1})
C	Dissolved oxygen concentration (mg L^{-1})
C_0	Initial dissolved oxygen concentration (mg L^{-1})
C_b	Baseline dissolved oxygen concentration (mg L^{-1})
C_f	Final dissolved oxygen concentration (mg L^{-1})
C_{LSto}	Storage baseline for oxygen concentration (mg L^{-1})
COD	Chemical oxygen demand (mg L^{-1})
CSTR	Continuous stirred tank reactor
D	Dilution rate (h^{-1})
DNS	Dinitrosalicylic acid
DO	Dissolved oxygen (mg O_2)
HRT	Hydraulic retention time (h)
k_La	Volumetric mass transfer coefficient (h^{-1})
K_S	Monod and ASM1 affinity constant (mg COD L^{-1})
K'_S	ASM3 affinity constant for storage material (mg COD L^{-1})

k_{Sto}	Storage rate constant (g S g^{-1} X d^{-1})
K_{Sto}	Affinity constant for storage material (mg COD L^{-1})
n	Number of experimental replicates
OUR	Oxygen uptake rate (mg L^{-1} h^{-1})
OUR_{ex}	Exogenous oxygen uptake rate (mg L^{-1} h^{-1})
$OUR_{ex}max$	Maximum exogenous oxygen uptake rate (mg L^{-1} h^{-1})
r^2	Correlation coefficient
S	Substrate concentration, measured as soluble COD (mg COD L^{-1})
S_E	Standard error
S_p	Pulse concentration injected (mg COD L^{-1})
TK	Tukey-Kramer test
tr	Response time (h)
X	Biomass concentration, measured as insoluble COD (mg COD L^{-1})
$Y_{O_2/S}$	Oxidation yield (mg COD mg^{-1} COD)
$Y_{Sto/S}$	Storage yield (mg COD mg^{-1} COD)
$Y_{X/S}$	Growth yield (mg COD mg^{-1} COD)
$Y'_{X/S}$	ASM3 growth yield (mg COD mg^{-1} COD)
$Y_{X/Sto}$	Storage growth yield (mg COD mg^{-1} COD)

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