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ON-LINE METABOLIC FLUX ANALYSIS IN A PHB PRODUCTION PROCESS

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Abstract: A model-based algorithm is presented for the on-line monitoring of the oxidative phosphorylation efficiency and intracellular metabolic fluxes in mixed microbial cultures producing Polyhydroxybutyrate (PHB). The method assumes the knowledge of the metabolic reactions and the respective material and energetic balances. The on-line availability of dissolved O_2 , dissolved CO_2, pH and off-gas concentrations of O_2 and CO2 provides a sufficient set of measurements for the estimation of the remaining fluxes. The estimator was evaluated with simulations. *Copyright © 2007 IFAC*

Keywords: Closed-loop control, Dynamic modelling, Estimators, Mathematical models, Model-based control, On-line control.

1. INTRODUCTION

Monitoring and control are key factors for achieving high productivity, robustness and reproducibility in bioprocesses. In mixed microbial systems, population dynamics are an important source of perturbation. The implementation of advanced process monitoring and control are extremely important for population selection, thereby improving batch consistency.

For mixed cultures, the use of on-line sensors is more restricted by the formation of cellular aggregates. Nevertheless, variables such as dissolved O_2 and CO_2, pH and off-gas composition can be easily measured on-line using standard sensors/measurement systems. Several papers have presented software sensors based on mathematical models and easily accessible measurements with the goal of estimating key culture parameters (yields, concentrations and kinetics) that are difficult to measure on-line (Farza *et al.*, 1997, Lubenova *et al.*, 2003 and Oliveira *et al.*, 2004 and 2005).

With the progress in the "-omic" sciences, the design of software sensors based on detailed metabolic information is becoming an attractive tool for the on-line monitoring of cellular metabolic activity, and in particular, for the on-line monitoring of the fluxome.

For the case of PHB production by mixed cultures studied in this work, an established metabolic model is available (van Aalst-van Leeuwen *et al.*, 1997, Dias *et al.*, 2005). This metabolic model is based on a set of six reactions, which describe the main processes involved in cellular metabolism. The corresponding theoretical yields and maintenance coefficients were derived from these reactions as functions of the oxidative phosphorylation efficiency (van Aalst-van Leeuwen *et al.*, 1997). The theoretical and experimental yields were compared by Dias *et al.* (2005), using experiments performed in a sequencing batch reactor (SBR) (Serafim *et al.*, 2004). The model assumes explicit "feast" and "famine" periods and was validated under such conditions.

In this paper, an on-line software sensor for this process, under the "feast" and "famine" operating regimen is proposed. The software sensor estimates on-line the key metabolic activity parameters as well as the concentrations of the most important compounds of this process.

2. METABOLIC MODEL

2.1 Metabolic reactions

Dias *et al.* (2005) proposed a metabolic model for PHB production by mixed cultures adapted from that by van Aalst-van Leeuwen *et al.* (1997) for *Paracoccus pantotrophus*. The model includes six reactions (Table 1) describing the main processes involved in the PHB production process. This process is operated in two distinct phases: the "feast" and "famine" phases. In the "feast" phase, acetate is consumed as the carbon source and in the "famine" phase, PHB is used as internal substrate.

Table 1. Metabolic model for PHB production by mixed cultures

Process description	Reactions
Acetate uptake	$CH_2O + ATP \rightarrow CHO_{0.5} + \frac{1}{2}\cdot H_2O$
Biomass synthesis	$1.267\cdot CHO_{0.5} + 0.2\cdot NH_3 + \left(K_{ATP} + \frac{m_{ATP}}{\mu}\right)\cdot ATP + 0.3\cdot H_2O \rightarrow CH_{1.4}N_{0.2}O_{0.4} + 0.533\cdot NADH_2 + 0.267\cdot CO_2$
Catabolism	$CHO_{0.5} + \frac{3}{2}\cdot H_20 \rightarrow CO_2 + 2\cdot NADH_2 + 0.5\cdot ATP$
Oxidative phosphorylation	$NADH_2 + \frac{1}{2}\cdot O_2 \rightarrow H_2O + \delta\cdot ATP$
PHB storage	$CHO_{0.5} + \frac{1}{4}\cdot NADH_2 \rightarrow CH_{1.5}O_{0.5}$
PHB consumption	$CH_{1.5}O_{0.5} + \frac{1}{4}\cdot ATP \rightarrow CHO_{0.5} + \frac{1}{4}\cdot NADH_2$

2.2 Yield and maintenance coefficients

A detailed stoichiometric analysis was performed by formulating balance equations of carbon, $NADH_2$, ATP and acetyl-CoA. These balances form a set of algebraic equations which were manipulated in order to obtain the theoretical yields and maintenance coefficients as functions of the oxidative phosphorylation efficiency (δ), biomass polymerization constant (K_{ATP}) and maintenance on ATP (m_{ATP}). The final results are presented in Table 2.

Table 2. Theoretical yields coefficients

Cell growth	PHB production
"Feast" phase	
$Y_{X/S} = \frac{4\cdot\delta - 1}{4\cdot\delta + 2\cdot K_{ATP} + 1.267}$	$Y_{P/S} = \frac{4\cdot\delta - 1}{4.5\cdot\delta + 1}$
$Y_{X/N} = 5$	-
$Y_{X/O}^{FEAST} = \frac{4\cdot\delta - 1}{2\cdot K_{ATP} + 2.267}$	$Y_{P/O} = \frac{4\cdot\delta - 1}{2.125}$
$Y_{X/C}^{FEAST} = \frac{4\cdot\delta - 1}{2\cdot K_{ATP} + 2.267}$	$Y_{P/C} = \frac{4\cdot\delta - 1}{0.5\cdot\delta + 2}$
"Famine" phase	
$Y_{X/P} = \frac{4.5\cdot\delta + 0.5}{4\cdot\delta + 2\cdot K_{ATP} + 1.267}$	-
$Y_{X/O}^{FAMINE} = \frac{4.5\cdot\delta + 0.5}{2.25\cdot K_{ATP} + 0.925}$	-
$Y_{X/C}^{FAMINE} = \frac{4.5\cdot\delta + 0.5}{-0.5\cdot\delta + 2\cdot K_{ATP} + 0.767}$	-

where $Y^k_{i/j}$ are the theoretical yield coefficients of component 'i' on component 'j' in the 'k' phase.

2.3 Process dynamics

The dynamic model for a batch reactor is obtained from mass balances for acetate, ammonia, PHB, biomass, H^+, O_2 and CO_2. The reactor is assumed to be well mixed and the gas phase is assumed to have a uniform composition. In terms of component concentrations, the matrix form of the model can be written as follows:

a) "Feast" phase

$$\frac{d}{dt}\begin{bmatrix} S \\ N \\ PHB \\ X \\ H^+ \\ O_2 \\ CO_2 \end{bmatrix} = K_{FEAST}\cdot\begin{bmatrix} q_{S,X} \\ q_{S,PHB} \\ m_S \end{bmatrix}\cdot X - \begin{bmatrix} 0 \\ 0 \\ 0 \\ 0 \\ n\cdot CTR \\ -OTR \\ CTR \end{bmatrix} \quad (1)$$

b) "Famine" phase

$$\frac{d}{dt}\begin{bmatrix} S \\ N \\ PHB \\ X \\ H^+ \\ O_2 \\ CO_2 \end{bmatrix} = K_{FAMINE}\cdot\begin{bmatrix} q_{PHB,X} \\ m_{PHB} \end{bmatrix}\cdot X - \begin{bmatrix} 0 \\ 0 \\ 0 \\ 0 \\ n\cdot CTR \\ -OTR \\ CTR \end{bmatrix} \quad (2)$$

where K_k is the matrix of yield coefficients in 'k' phase, OTR is the oxygen transfer rate, CTR is the carbon dioxide transfer rate and n is the number of protons produced from each mol of CO_2 released by cell respiration. The oxygen transfer rate is defined as $K_La\cdot(C_{O2}^{*}-C_{O2})$, where K_La is the global mass transfer coefficient, C_{O2}^{*} is the saturation concentration both in the liquid phase and C_{O2} is the dissolved oxygen concentration. The carbon dioxide transfer rate is defined as $0.2\cdot K_La\cdot C_{CO2}$, where C_{CO2} is the dissolved carbon dioxide concentration.

The model is comprised of two partial models (for the "feast" and "famine" phases). In the "feast" phase, acetate uptake for growth, PHB production and maintenance ($q_{S,X}$, $q_{S,PHB}$ and m_S) occur. In the "famine" phase the PHB previously produced is consumed for growth and maintenance ($q_{PHB,X}$ and m_{PHB}). These two states never occur simultaneously and the switch between the "feast" and "famine" phase takes place after acetate depletion.

Pratt *et al.* (2004) reported the three main processes directly affecting the H^+ accumulation in the liquid phase as being acetate and ammonia uptake and CO_2 production and transfer. In the liquid phase, the acetic acid is in equilibrium with acetate. Assuming that cells consume the substrate in undissociated form, they assimilate also H^+ from the liquid phase during the carbon uptake, causing the pH to increase. m is the mmol of H^+ consumed in the acetate acid/base

equilibrium reaction and can be expressed as a function of the pH and the pK_A of the acetic acid equilibrium reaction (eq. 3).

$$m = \frac{10^{-pK_A}}{10^{-pH} + 10^{-pK_A}} \tag{3}$$

The CO_2 released during the cell respiration is transferred to the liquid phase and contributes to H^+ production through the bicarbonate/carbonate equilibrium reactions. n is the amount of H^+ produced per CO_2 molecule released to the liquid phase, and is dependent on pH and the equilibrium constants $pK_{CO2,1}$ and $pK_{CO2,2}$ (eq. 4).

$$n = \frac{2 \cdot 10^{2 \cdot pH} + 10^{(pH + pK_{CO2,2})}}{10^{2 \cdot pH} + 10^{(pH + pK_{CO2,2})} + 10^{(pK_{CO2,1} + pK_{CO2,2})}} \tag{4}$$

The nitrogen is incorporated into the new biomass in the form of NH_3. In the liquid phase, NH_3 is in equilibrium with NH_4^+. The NH_4^+ fraction, p, is dependent on pH (eq. 5). pK_{NH4} is the acid-base equilibrium constant for ammonia.

$$p = \frac{10^{-pH}}{10^{-pH} + 10^{-pK_{NH4}}} \tag{5}$$

Knowing the stoichiometry for the seven state variables, the yield coefficient matrices for the "feast" and "famine" phases can be represented as follows.

a) "Feast" phase

$$K_{FEAST} = \begin{bmatrix} -1 & -1 & -1 \\ -\frac{Y_{X/S}}{Y_{X/N}} & 0 & 0 \\ 0 & Y_{P/S} & 0 \\ Y_{X/S} & 0 & 0 \\ m + p \cdot \frac{Y_{X/S}}{Y_{X/N}} + n \cdot \frac{Y_{X/S}}{Y_{X/C}^{FEAST}} & m + n \cdot \frac{Y_{P/S}}{Y_{P/C}^{FEAST}} & n - m \\ -\frac{Y_{X/S}}{Y_{X/O}^{FEAST}} & -\frac{Y_{P/S}}{Y_{P/O}^{FEAST}} & -1 \\ \frac{Y_{X/S}}{Y_{X/C}^{FEAST}} & \frac{Y_{P/S}}{Y_{P/C}^{FEAST}} & 1 \end{bmatrix} \tag{6}$$

b) "Famine" phase

$$K_{FAMINE} = \begin{bmatrix} 0 & 0 \\ -\frac{Y_{X/P}}{Y_{X/N}} & 0 \\ -1 & -1 \\ Y_{X/P} & 0 \\ p \cdot \frac{Y_{X/P}}{Y_{X/N}} + n \cdot \frac{Y_{X/P}}{Y_{X/C}^{FAMINE}} & n \\ -\frac{Y_{X/P}}{Y_{X/O}^{FAMINE}} & -1.125 \\ \frac{Y_{X/P}}{Y_{X/C}^{FAMINE}} & 1 \end{bmatrix} \tag{7}$$

3. DESIGN OF AN ESTIMATOR FOR ONLINE METABOLIC FLUX ANALYSIS

The estimator structure assumes the existence of two partial models of the "feast" and "famine" phases, thus yielding two partial algorithms. The transition between these two states is detected by the depletion of acetate.

There are three unknown reaction rates in the "feast" phase and two in the "famine" phase. In the "feast" phase, the unknown reaction rates are the specific acetate uptake rates for growth, PHB production and maintenance. In the "famine" phase, the PHB uptake rates for growth and maintenance are not known. Throughout the process, the dissolved and off-gas concentrations of O_2 and CO_2, as well as the H^+ concentration in the liquid phase can be measured on-line through physical sensors. Thus, the number of unknown reaction rates is lower ("famine" phase) or equal ("feast" phase) to the number of measured state variables.

The estimation of the unknown reaction kinetics in systems (1) and (2) can be performed using an observer-based estimator (Bastin and Dochain, 1990). The observer-based estimator assumes full knowledge of the yield matrices. In the problem studied here, the yield coefficients may vary in time depending on the dynamics of the efficiency of oxidative phosphorylation, δ. This dependency has been previously established from the material and energy balances applied to the metabolic reactions (eqs. 6-7) given that K_{ATP} and m_{ATP} are fixed. In practice, the estimation problem is further complicated by the additional time-varying parameter, δ. This parameter may, however, be estimated from the reaction kinetics using the following two equations, which were derived from the overall ATP, $NADH_2$ and acetyl-CoA balances in the "feast" and "famine" phases:

a) "Feast" phase

$$\hat{\delta} = \frac{\left(\hat{q}_{S,X} + \hat{q}_{S,PHB} + \hat{m}_S + 4.67 \cdot \hat{Y}_{X/S} \cdot \hat{q}_{S,X} + \hat{Y}_{P/S} \cdot \hat{q}_{S,PHB} + 2 \cdot m_{ATP}\right)}{\left[4 \cdot \left(\hat{q}_{S,X} + \hat{q}_{S,PHB} + \hat{m}_S\right) - 4 \cdot \hat{Y}_{X/S} \cdot \hat{q}_{S,X} - 4.5 \cdot \hat{Y}_{P/S} \cdot \hat{q}_{S,PHB}\right]} \tag{8}$$

b) "Famine" phase

$$\hat{\delta} = \frac{\left[-0.5 \cdot \left(\hat{q}_{PHB,X} + \hat{m}_{PHB}\right) + 4.67 \cdot \hat{Y}_{X/P} \cdot \hat{q}_{PHB,X} + 2 \cdot m_{ATP}\right]}{\left[4.5 \cdot \left(\hat{q}_{PHB,X} + \hat{m}_{PHB}\right) - 4 \cdot \hat{Y}_{X/P} \cdot \hat{q}_{PHB,X}\right]} \tag{9}$$

Note that the "true" reaction rates and yields were replaced by their estimates.

The application of observer-based estimator to systems (1) and (2) taking H^+, O_2 and CO_2 as measurable variables, results in the following equations:

$$\begin{cases} \frac{d\xi^{MEAS}}{dt} = K^{MEAS}\left(\hat{\delta}\right) \cdot \hat{\rho} + Q^{MEAS} - \omega_1 \cdot \left(\xi^{MEAS} - \hat{\xi}^{MEAS}\right) \\ \frac{d\hat{\rho}}{dt} = \omega_2 \cdot \left(\xi^{MEAS} - \hat{\xi}^{MEAS}\right) \end{cases} \tag{10}$$

where $K^{MEAS}\left(\hat{\delta}\right)$ is the yield coefficient matrix corresponding to H^+, O_2 and CO_2 (eqs. 6-7) for a given δ estimate, $\hat{\delta}$, ξ^{MEAS} and $\hat{\xi}^{MEAS}$ are the measured and estimated concentrations of H^+, O_2 and CO_2 in the liquid phase, $\hat{\rho}$ is the estimated reaction rates vector and Q^{MEAS} is the mass transfer rate term

between the gas and liquid phases, which is assumed to be measured on-line:

$$\hat{\rho}_{FEAST} = \begin{bmatrix} \hat{q}_{S,X} \\ \hat{q}_{S,PHB} \\ \hat{m}_S \end{bmatrix} \quad \hat{\rho}_{FAMINE} = \begin{bmatrix} \hat{q}_{PHB,X} \\ \hat{m}_{PHB} \end{bmatrix} \quad Q^{MEAS} = \begin{bmatrix} -n \cdot CTR \\ OTR \\ -CTR \end{bmatrix} \tag{11}$$

The system represented by eq. (10) has a second order time-invariant convergence response (Oliveira *et al.*, 1994, 1995, 2002) as long as the following calibration rules (for tunning ω_1 and ω_2) are obeyed:

$$\omega_1 = 2 \cdot \frac{\zeta}{\tau} \tag{12}$$

$$\omega_2 = \frac{1}{\tau^2} \cdot \left[K^{MEAS}\right]^{-1} \tag{13}$$

with τ and ζ are the natural periods of oscillation and damping coefficients of the second order dynamical response.

From the estimated reaction rates, the dynamics of the unmeasured concentration of acetate, biomass, PHB and ammonia can be easily obtained from the corresponding material balances:

a) "Feast" phase

$$\frac{d}{dt}\begin{bmatrix} \hat{S} \\ \hat{PHB} \\ \hat{X} \\ \hat{N} \end{bmatrix} = K_{FEAST}^{UNMEAS}\left(\hat{\delta}\right) \cdot \begin{bmatrix} \hat{q}_{S,X} \\ \hat{q}_{S,PHB} \\ \hat{m}_S \end{bmatrix} \cdot \hat{X} - \begin{bmatrix} 0 \\ 0 \\ 0 \\ 0 \end{bmatrix} \tag{14}$$

b) "Famine" phase

$$\frac{d}{dt}\begin{bmatrix} \hat{S} \\ \hat{PHB} \\ \hat{X} \\ \hat{N} \end{bmatrix} = K_{FAMINE}^{UNMEAS}\left(\hat{\delta}\right) \cdot \begin{bmatrix} \hat{q}_{PHB,X} \\ \hat{m}_{PHB} \end{bmatrix} \cdot \hat{X} - \begin{bmatrix} 0 \\ 0 \\ 0 \\ 0 \end{bmatrix} \tag{15}$$

4. RESULTS AND DISCUSSION

4.1 Simulation model

For simulation purpose, the kinetic model proposed by Dias *et al.* (2005) was adopted (see Table 3). This model assumes explicitly a "feast" and a "famine" phase. In the "feast" phase, acetate is driven simultaneously to biomass growth (limited by ammonia), PHB production and maintenance. After the external substrate depletion, the process state is switched to the "famine" phase, and the PHB previously stored is used for biomass growth and maintenance. This model was validated with five calibration batch experiments and three prediction batch experiments performed by Serafim *et al.*, 2004.

Table 3. Kinetic model for PHB production by mixed cultures

	Process	Kinetic equations
"Feast" phase	Growth	$q_{S,X} = q_{SX,max} \frac{S}{S+K_S} \frac{N}{N+K_{N,S}} \frac{O}{O+K_O}$
	PHB Production	$q_{S,PHB} = q_{SPHB,max} \frac{S}{S+K_S} \frac{O}{O+K_O} \left[1 - \left(\frac{f_{PHB}}{f_{PHB,max}}\right)^{\alpha}\right]$
	Maintenance	$m_S = m_{S,max} \cdot \frac{S}{S+K_S} \frac{O}{O+K_O}$ $\quad m_{S,max} = \frac{2 \cdot m_{ATP}}{4 \cdot \delta - 1}$
"Famine" phase	Growth	$q_{PHB,X} = q_{PHBX,max} \frac{f_{PHB}}{f_{PHB}+K_{PHB}} \frac{N}{N+K_{N,PHB}} \frac{O}{O+K_O} \frac{K_I}{S+K_I}$
	Maintenance	$m_{PHB} = k \cdot fPHB^{nPHB} \frac{O}{O+K_O} \frac{K_I}{S+K_I}$

where S is acetate, f_{PHB} and $f_{PHB,max}$ are the intracellular polyhydroxybutyrate fraction and its maximum value, N is ammonia, and O is oxygen. $q_{j,k}$, $q_{jk,max}$, m_j and $m_{i,max}$ are specific reaction rates and their maximum coefficients. $K_{j,k}$ and K_j are half-saturation constants. K_I is the acetate inhibition constant. k is the kinetic constant for PHB degradation. n_{PHB} is the reaction order of PHB degradation for maintenance. α is the PHB production saturation order constant.

The equations for oxygen and carbon dioxide dynamics are derived from the main process kinetics and from the respective yield coefficients.

a) "Feast" phase

$$OUR = \frac{Y_{X/S}}{Y_{X/O}^{FEAST}} \cdot q_{S,X} + \frac{Y_{P/S}}{Y_{P/O}^{FEAST}} \cdot q_{S,PHB} + m_S \tag{16}$$

$$CPR = \frac{Y_{X/S}}{Y_{X/C}^{FEAST}} \cdot q_{S,X} + \frac{Y_{P/S}}{Y_{P/C}^{FEAST}} \cdot q_{S,PHB} + m_S \tag{17}$$

b) "Famine" phase

$$OUR = \frac{Y_{X/S}}{Y_{X/O}^{FAMINE}} \cdot q_{PHB,X} + m_S \tag{18}$$

$$CPR = \frac{Y_{X/S}}{Y_{X/C}^{FAMINE}} \cdot q_{PHB,X} + m_S \tag{19}$$

The H^+ production rate (HPR) can be written in terms of the main process reactions of the "feast" and "famine" phases as follow:

a) "Feast" phase

$$HPR = \left(p \cdot \frac{Y_{X/S}}{Y_{X/N}} + n \cdot \frac{Y_{X/S}}{Y_{X/C}^{FEAST}} - m\right) \cdot q_{S,X} + \left(n \cdot \frac{Y_{P/S}}{Y_{P/C}^{FEAST}} - m\right) \cdot q_{S,PHB} + (n-m) \cdot m_S \tag{20}$$

b) "Famine" phase

$$HPR = \left(p \cdot \frac{Y_{X/P}}{Y_{X/N}} + n \cdot \frac{Y_{X/P}}{Y_{X/C}^{FAMINE}} - m\right) \cdot q_{PHB,X} + n \cdot m_{PHB} \tag{21}$$

4.2 Assessment of estimator performance

The estimator algorithm used in this study was tested with simulated data obtained using the kinetic model described above. The simulations were carried out with the following initial conditions and the model parameters listed in Table 4. The process was simulated at a constant pH of 7.

$S(0) = 200$ C-mmol·L^{-1} $\quad N(0) = 10$ N-mmol·L^{-1}
$PHB(0) = 7$ C-mmol·L^{-1} $\quad X(0) = 70$ C-mmol·L^{-1}
$H^+(0) = 1 \times 10^{-7}$ mol·L^{-1} $\quad O_2(0) = 0.22$ mmol·L^{-1}
$CO_2(0) = 0$ mmol·L^{-1}

Table 4. Parameter values

Constants	Value	Units	Constants	Value
$q_{SX,max}$	0.18	C-mol.(C-mol.h)$^{-1}$	α	4.72
$q_{SPHB,max}$	0.70	C-mol.(C-mol.h)$^{-1}$	n_{PHB}	2.03
m_{ATP}	0.02	mol-ATP.(C-mol.h)$^{-1}$	pK_A	4.75
$q_{PHBX,max}$	0.097	C-mol.(C-mol.h)$^{-1}$	$pK_{CO2,1}$	6.30
$f_{PHB,max}$	2.54	C-mol.C-mol^{-1}	$pK_{CO2,2}$	10.30
k	0.067	C-mol.(C-mol.h)$^{-1}$	pK_{NH4}	9.25
K_{ATP}	1.70	mol ATP.C-mol^{-1}		
K_S, K_I	0.062	C-mmol·L^{-1}		
$K_{N,S}$, $K_{N,PHB}$	0.59	N-mmol·L^{-1}		
K_O	0.019	mmol.L^{-1}		
K_{PHB}	0.0001	C-mol.(C-mol.h)$^{-1}$		
$Y_{X/N}$	0.2	C-mol.(N-mol)$^{-1}$		

The process model and estimator were implemented in a MATLAB^{TM} program. The simulation time was 12h. The initial ammonia concentration was tuned in order to promote a certain amount of cell growth during the "famine" phase. In this study, it is assume that the metabolic reactions are correct but that the efficiency of oxidative phosphorylation is unknown. The oxidative phosphorylation efficiency was intentionally perturbed every two hours between 2.91 and 1.5, according to a square wave signal. The process dynamics is shown in Fig. 1 (simulated state variables represented as full lines). Normally distributed noise, with standard deviation of 2 % and zero mean, was added to the simulated concentrations to mimic real noisy measurements.

The estimator ran using the simulated process data with sampling time of 3.6 seconds. Constant values of the damping factor (ζ) and the natural period of oscillation (τ) were used throughout the simulations, using the tuning eqs. (12-13). Fig. 1 shows the results obtained with $\zeta = 1$ and $\tau = 6$ min. The solid lines represent simulated values for the key variables and the dashed lines represent their estimation. The oxidative phosphorylation efficiency (δ) imposed during the process simulation is also presented in Fig. 1.

In the transition between the "feast" and the "famine" phases, oscillations are observed in the predictions that affect mainly the estimation of the biomass and ammonia concentrations. However, the overall results show a good agreement between the simulated and estimated unmeasured state variables for both process phases.

Table 5. Estimated and "true" reaction rates for t = 1 h

Process	Estimated rates [C-mol.(C-mol)$^{-1}$]	"True" rates [C-mol.(C-mol)$^{-1}$]	Error (%)
Acetate uptake	0.73	0.72	0.32
Biomass synthesis	0.066	0.066	1.26
Catabolism	0.24	0.25	4.20
Oxidative phosphorylation	0.45	0.48	6.09
PHB storage	0.39	0.37	4.24

Table 5 shows several (potentially on-line) estimated fluxes at time t = 1 h. At this time instant the estimated and the "true" oxidative phosphorylation efficiency ($\hat{\delta}$) were 1.62 and 1.5, respectively. The estimated and "true" fluxes show a good agreement, thus highlighting the potential of this method for on-line metabolic fluxes monitoring.

5. CONCLUSIONS

In this work an on-line estimation algorithm of key metabolic parameters in a PHB producing mixed microbial culture was derived. The algorithm allows the on-line estimation of the efficiency of oxidative phosphorylation, δ, yield coefficients, intracellular fluxes and concentrations of unmeasured state variables from easily available measurements of pH, dissolved O_2 and CO_2 and off-gas composition. The algorithm assumes the knowledge of the metabolic reactions, but does not take δ as a constant. This issue is particularly important in mixed microbial cultures since the composition of the microbial population may vary in time. The estimator was evaluated with simulation studies, whereby the "true" δ and kinetics were hidden to the estimator. The simulation results clearly indicate that exponential convergence may be achieved for particular estimator tunings. The experimental validation is currently under way. Potential problems for the implementation of this algorithm are the possibility of unbalanced carbon due to the formation of (undesirable) exopolysaccharides and also possible interferences in the acid-base equilibrium. The correlation between pH and the amount of H^+ produced, HPR, must be quantified. Note that the determination of HPR by titrimetric measurements (Pratt *et al.*, 2004) implies a pH-stat. This may be however detrimental for the process productivity as demonstrated in Serafim *et al.* (2004).

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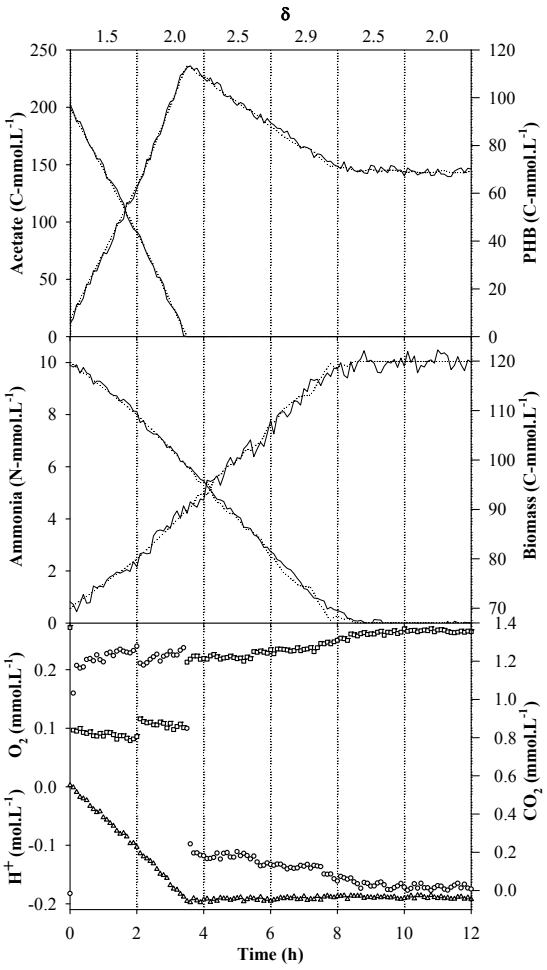

Fig. 1 Simulated and estimated results of acetate, PHB, ammonia and biomass concentration for the "feast" and "famine" phases. Solid lines represent simulated results and dashed lines represent estimated results. (Δ), (□) and (O) are the simulated hydrogen, oxygen and carbon dioxide concentrations, respectively.