J Therm Anal Calorim (2011) 104:75–85
DOI 10.1007/s10973-010-1259-x

Biocalorimetry as a process analytical technology process analyser; robust in-line monitoring and control of aerobic fed-batch cultures of crabtree-negative yeast cells

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ISBCXVI Special Issue

Abstract Control of bioprocesses requires reliable and robust on- or in-line monitoring tools providing real-time information on process dynamics. Heat generation related to metabolic activity of living systems is currently gaining importance in bioprocess industry due to its non-invasive and essentially instantaneous characteristics. This study deals with monitoring and control of pure aerobic fed-batch cultures of three Crabtree-negative yeast strains, *Kluyveromyces marxianus*, *Candida utilis* and *Pichia pastoris*, based on in-line measured, metabolic heat flow signals. A high resolution biocalorimeter (BioRC1) was developed from a standard bench-scale heat flow calorimeter (RC1). The BioRC1 was equipped with in-line (dielectric spectroscopy, pH probe and dissolved oxygen probe) and at-line (exit gas analyser) sensors to characterise the growth behaviour of the yeast cells. Both metabolic heat flow and biomass profiles exhibited similar behaviour proving the significance of employing heat flow signal as a key-parameter for the system under investigation. A simple estimator for biomass concentration and specific growth rate was formulated based on heat flow values. In order to evaluate the potential of calorimetry as a reliable and powerful process monitoring tool, the robustness, reliability as well as the broad applicability of the developed estimators was assessed through comparison with off-line measurement techniques and showed promising results for general applicability with a wide range of bioprocesses.

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Keywords Biocalorimetry · Bioprocess control · PAT bioprocess monitoring · Fed-batch cultures

List of symbols

A	Wetted heat exchange area (m^2)
$C_{p,\text{base}}$	Specific heat capacity of the base (kJ kg^{-1} K^{-1})
$C_{p,\text{r}}$	Specific heat capacity of reaction broth (kJ kg^{-1} K^{-1})
$C_{p,\text{substrate}}$	Specific heat capacity of the substrate feed solution (kJ kg^{-1} K^{-1})
m_{base}	Mass flow rate of the base (g h^{-1})
$m_{\text{substrate}}$	Mass flow rate substrate feed solution (g h^{-1})
q_{b}	Baseline heat flow rate (W)
q_{cal}	Calibration power (W)
q_{s}	Heat production rate due to stirring (W)
q_{f}	Heat flow rate through the reactor wall (W)
q_{g}	Heat flow rate caused by the aeration (W)
q_{d}	Heat flow rate due to dosing (W)
q_{l}	Heat flow rate through the reactor head assembly by radiation and conduction (W)
q_{r}	Heat flow rate generated due to metabolic activity of the cells (W)
Q_0	Cumulative metabolic heat production at the end of batch phase (kJ)
Q_t	Cumulative metabolic heat production at time t (kJ)
r_i	Rate of production or consumption of component i (g L^{-1} h^{-1})
r_x	Rate of biomass generation (g L^{-1} h^{-1})
t	Process time (h)
T_{a}	Ambient temperature (K)
T_{base}	Temperature of the base (K)
T_r	Reaction temperature (K)

$T_{substrate}$	Temperature of the substrate feed solution (K)
T_j	Jacket temperature (K)
U	Overall heat transfer coefficient of the reactor wall (W m^{-2} K^{-1})
V_0	Volume of the reaction broth at end of batch phase (L)
V_r	Volume of reaction broth (L)
x	Biomass concentration (g L^{-1})
x_0	Initial biomass concentration (g L^{-1})
x_t	Biomass concentration at time t (g L^{-1})
$Y_{Q/i}$	Heat yield due to production or consumption of component i (kJ g^{-1})
$Y_{Q/x}$	Heat yield due to biomass growth (kJ g^{-1})
α	heat loss coefficient (W K^{-1})
μ	Specific growth rate (h^{-1})
$\mu_{est,t}$	Specific growth rate at time t estimated based on heat rate measurements (h^{-1})

Introduction

Biotechnological processes are rather complex systems especially regarding effective monitoring and control. Control of biotechnological processes requires reliable real-time measurements of critical process parameters. During the last years several sensors and controllers have been developed and implemented for in-line monitoring and control of bioprocess systems [1]. Invasive mode of operation, time delay in measured signals and difficulties in integrating lab-scale tested systems into existing industrial bioprocesses are the major drawbacks of custom available sensors [2]. Recently, biocalorimetry has been found to have potential for real-time bioprocess monitoring due to its non-invasive and instantaneous mode of operation [3]. Indeed, heat signals during a bioprocess provide a global insight into metabolic activity of living cells [4, 5]. In the case of a pure aerobic respiratory metabolism where the substrate is entirely converted into biomass, water, carbon dioxide and heat, the signal measured by a biocalorimeter (BioRC1) can be related to the actual biomass concentration as well as to specific growth rate. Product quality, as well as the productivity of a process is influenced by the specific growth rate and its fluctuation. Controlling this critical process parameter is an important step towards quality by design as encouraged by the FDA initiative [6].

Several authors so far attempted to employ calorimetry as an analytical tool to monitor and investigate various bioprocesses [7], [8, 9]. However, literature related to applications of heat signal as a basis for control of the specific growth rate fed-batch cultures of yeast cells are scarce. While most reported research applied heat flow signals as an indicative measurement to start the nutrient feeding at the end of batch phase [10], the actual measurements used to control the substrate feed rate of the process were then heat-derived parameters such as the oxycalorific equivalent or combined parameters such as heat flow signals combined with to carbon dioxide evolution rate [11, 12].

In this study, a reliable biomass and specific growth rate estimator is developed based on heat flow measurements. The principle aim of the study is to highlight the importance of a creating biomass and specific growth rate estimator based on simple process parameter measurements. An additional step includes the improvement of the reliability and the robustness of the developed estimators in order to ensure, at a latter stage, an established basis for bioprocess control.

In accordance with classical biothermodynamics it is possible to use the calorimetric heat flow signal as a sole variable to control microbial fed-batch culture since, for pure aerobic bioprocesses involving Crabtree-negative organisms, the cell growth is entirely based on its respiratory capacity and further characterised by the absence of by-product formation. Moreover, the heat generation rate due to metabolic activity of living cells is directly proportional to the growth rate at any instant of time unless there is a substrate limitation [13]. This study is therefore centred on the aerobic batch and fed-batch cultures of Crabtree-negative yeasts, namely *Kluyveromyces marxianus*, *Candida utilis* and *Pichia pastoris*. These microbes were chosen as model organisms due to their simple growth requirements as well as to the fact that they do not suffer from any metabolic bottlenecks enabling the direct correlation between heat generation rate and specific growth rate in its simplest way. Model development and initial test were carried out with *K. marxianus*, while *C. utilis* and *P. pastoris* were used to test-proof the general applicability of the designed estimators to different Crabtree-negative yeast strains.

The development of a robust biomass and specific growth rate estimator based on heat-measurements has its importance in the framework of the FDA's PAT initiative, since it gives a real-time insight into the on-going industrial processes. In fact, it has been proven that small modifications to a standard large-scale bioreactor allow the acquisition of in-line calorimetric measurements [14]. The proposed biomass and specific growth rate estimator might therefore present a simple, but robust and non-invasive way of monitoring an industrial microbial bioprocess.

Experimental

Cell strains, culture conditions and off-line analysis

Cell strains

A wild-type yeast strain, *K. marxianus* (DSM 5422, DSMZ, Braunschweig, Germany) was chosen for this study, mainly due to its Crabtree-negative metabolism.

For the assessment of the general applicability of the developed estimators, two other yeast strains were cultured, namely *C. utilis* (DSM 2361, DSMZ, Braunschweig, Germany) and *P. pastoris* (PPC43AZ) provided by Dr. Andrea Zocchi from the University of Neuchâtel, Switzerland.

All strains were preserved as 1.8 mL aliquots in a 20 g L^{-1} glycerol solution at −80 °C.

Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA).

Inoculum preparation

The preculture was obtained by suspending cells from a 1.8 mL aliquot in a 1-L, baffled Erlenmeyer flask containing 100 mL of preheated, sterile, complex preculture medium (pH 4 for *C. utilis* and pH 5 for *K. marxianus* and *P. pastoris*) containing 20 g L^{-1} of glucose for *K. marxianus* and *C. utilis* and 20 g L^{-1} of glycerol for *P. pastoris*, 10 g L^{-1} of yeast extract and 10 g L^{-1} of peptone and incubating the flask in an orbital shaker incubator (SHEL LAB S19, Sheldon Manufacturing, Cornelius, USA) for 24 h at 30 °C and at 150 rpm. Ninety millilitres of the culture broth was then centrifuged at 3,000 rpm for 10 min, the supernatant discarded and the cell pellet suspended in 10 mL of a sterile saline solution (9 g L^{-1} of NaCl).

Reactor description, media composition and culture conditions

The preculture was used to inoculate a 2-L BioRC1 (modified RC1, Mettler Toledo, Greifensee, Switzerland) with a working volume of 1.3 L, equipped with a Rushton-type agitator, baffles, pH probe and controller, gas inlet and outlet ports, a base inlet port, a port for a capacitance probe (Biomass Monitor, ABER Instruments, Aberysthwyth, UK) and a sampling valve. The aeration rate was set to 2.5 L/min using a thermal massflow controller (5850E, Brooks, The Netherlands) and the air was sterilized by passage through a 0.22 μm filter before entering the biocalorimeter. Outlet gas was passed through a Wolff bottle followed by a 0.22 μm filter before entering a gas analyser (Duet, Applied BioSystems Ltd., UK). The measured values of O_2 and CO_2 were corrected for water vapour according Duboc and von Stockar [15] and used to evaluate in real-time the oxygen up-take rate (OUR), the carbon dioxide evolution rate (CER) as well as the respiratory quotient (RQ). A solution of 4 M NaOH was used to maintain the pH at 5. Acid control was not necessary due to the composition of the culture medium and the cell metabolism. The operating principle of the BioRC1 has been reported previously in literature [16]. All experiments were performed with a working volume of 1.3 L and the reaction temperature was maintained constant at 30 °C. Agitation rate was maintained at 800 rpm throughout all cultures. A detailed account of liquid volumes entering the reactor (base and medium feed) and leaving it (samples) was made gravimetrically (Analytical Balances, Mettler Toldedo, Greifensee, Switzerland) and filed through a LabVIEW (LabVIEW 8.2, National Instruments, Austin, USA) program in order to have a continuous inventory of the reactor volume throughout the experiment.

Figure 1 depicts a schematic representation of the experimental set-up including all the instruments, their accessories and their connections.

Prior to inoculation, the bioreactor was sterilized in situ at 121 °C for 20 min with de-ionised water using an automated WINRC sterilisation program (WINRC Software, Mettler Toledo, Greifensee, Switzerland), cooled down at room temperature, drained and filled with sterile batch medium. Cells were grown in batch mode until depletion of the carbon and energy source. For the assessment of the growth rate estimator, a fed-batch culture of *K. marxianus* was carried out, where an exponential feed mode, described elsewhere [17] was started after the end of the batch phase. Tables 1 and 2 outline the composition of the defined batch and feed media. The media were prepared with de-ionised water, sterilized by filtration (Steritop, 0.22 μm pore size, Millipore, Billerica, USA) and supplemented with sterile antifoam solution, trace elements and vitamins as prescribed by Verduyn et al. [18] and Cannizzaro et al. [19] for *K. marxianus* and *C. utilis* and suggested by Invitrogen for *P. pastoris*.

Monitoring, data acquisition and control strategy

Off-line analysis

Samples (10 mL) were taken at regular intervals during the cultures in order to determine off-line the biomass concentration. Biomass dry cell weight (DCW) was determined by filtering a known volume of the culture broth through a pre-weighed 0.22 μm pore size filter (GSWP 0.22 μm Nitrocellulose membrane filters, Millipore,

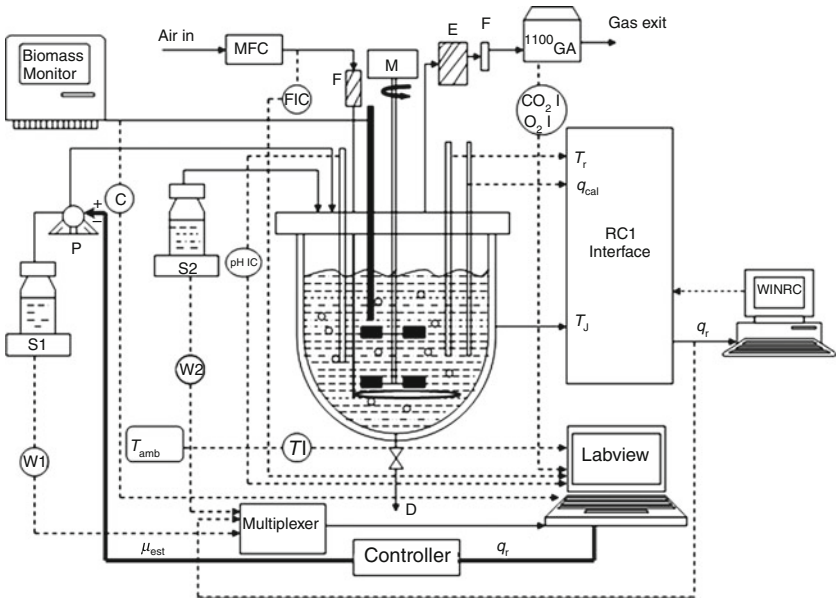

Fig. 1 Schematic representation of the experimental set-up around the BioRC1system: *C* Capacitance, *D* Drain valve, *E* Entrainer, *F* Membrane Filter, *GA* Gas analyzer, *M* Motor, *MFC* Mass flow controller, *P* Peristaltic pump, *S1*, *S2* Balances, T_{amb} Ambient temperature probe, T_r Reaction temperature, T_j Jacket temperature, q_{cal} Calibration heat flow, q_r Net metabolic heat flow, *W1*, *W2*; Mass, μ_{est} Estimated growth rate,. *Dotted lines* represent the in-line measured values for LABVIEW. *Double line* represents the estimated control parameter from LABVIEW

Table 1 Composition of the batch and feed media for culturing *K. marxianus* and *C. utilis*

Component	Batch medium/gL^{-1}	Feed medium/gL^{-1}
Glucose	10	300
$(NH_4)_2SO_4$	5	50
KH_2PO_4	3	35
$MgSO_4{\cdot}7H_2O$	0.5	3
Component	Batch medium/mL L^{-1}	Feed medium/mL L^{-1}
Trace elements and vitamins solution	5	15
Polypropylenglycol (Antifoam)	0.5	2

Table 2 Composition of the batch and feed media for culturing *P. pastoris*

Component	Batch medium/gL^{-1}	Feed medium/gL^{-1}
Glycerol	10	500
$CaS0_4{\cdot}2H_2O$	0.59	–
K_2SO_4	9.1	–
$MgSO_4{\cdot}7H_2O$	7.45	–
KOH	2.06	–
NH_4Cl	9	45
Component	Batch medium/mL L^{-1}	Feed medium/mL L^{-1}
H_3PO_4 85%	13.35	–
Trace elements and vitamins solution	4.35	–
Polypropylenglycol (Antifoam)	1	–

Billerica, USA), drying the filter to constant weight and subsequently reweighing.

Data acquisition and process control

Data acquisition of the process parameters from the different probes and sensors was carried out through a Field Point (Model—FP 2000, National Instruments, USA) and an interfacing hardware (NB-MID-32X, National Instruments) with a PC. A LabVIEW program (LabVIEW 8.2, National Instruments, Austin, USA) was developed in-house for data acquisition, storage and display as well as for calibration and process control. The acquired raw values, saved in a separate file, were continuously averaged over 50 points and used for the real-time calculations of the baseline heat flow, the metabolic heat flow, the biomass concentration, the specific growth rate, the nutrient feed rate and the error term for the feedback control loop for the control strategy under development.

Biomass and specific growth rate estimator

Specific growth rate estimators are commonly based on Monod-derived models, requiring reliable direct biomass measurements such as capacitance measurements [20] or indirect estimations based on oxygen up-take rate [21]. However, a heat-yield based approach may be employed as stated by von Stockar and van der Wielen [22]. The detailed development for the heat-based biomass estimator and the specific growth rate estimator for fed-batch

cultures, described in Eqs 1 and 2 may be found in the appendix.

$$x_t \cdot V_t = x_0 \cdot V_0 + \frac{Q_t - Q_0}{Y_{Q/X}} \quad (1)$$

Equation 1 describes the estimation of biomass at a given time t as a function of the cumulative heat release and the heat yield coefficient.

$$\mu_{\text{est},t} = \frac{q_r}{x_0 \cdot V_0 \cdot Y_{Q/X} + (Q_t - Q_0)} \quad (2)$$

Equation 2 describes the estimation of the specific growth rate as a function of the initial biomass concentration, the heat yield coefficient, the cumulative heat production and the heat production rate.

The drawback of instantaneous estimators is their extreme vulnerability to noise in the values serving as a basis for the estimation. Therefore, the measurements need to be smoothed by applying for instance moving-point averaging or mathematical filtering techniques. Throughout this study, a moving-point average of 50 points was applied systematically to all raw data. Several different moving-point averages were applied and the influence on the stability of the measurements studied. The results indicated that 50 points seemed to be a good compromise between stability and robustness of the measurements without losing vital information through over-smoothing of the signal.

Results and discussion

This study can be divided into four different parts. In a first step, the different on-, in- and at-line monitoring techniques included in the reactor setup, as described in "Experimental" were qualitatively evaluated in terms of sensitivity to noise and information provided. In addition, correlation studies, relating the heat flow rate to different off-line biomass measurements, were conducted to justify the use of calorimetric data as a basis of the development of a reliable biomass and specific growth rate estimator for Crabtree-negative yeast cultures and experimental heat yield coefficient were inferred from batch cultures of the different strains. In a third part, the biomass estimator based on heat measurements is assessed regarding reliability and robustness by comparing the results to off-line dry cell weight measurements and the specific growth rate estimator was also tested in a fed-batch experiments using a control strategy developed in previous study [17]. In a final step, the proposed biomass estimator was tested on two different Crabtree-negative yeast strains, *C. utilis* and *P. pastoris*, under different culture conditions in terms of pH and medium composition namely.

Qualitative evaluation and comparison of different monitoring techniques

Figure 2 depicts comparative profiles of heat flow rate, capacitance, oxygen up-take rate (OUR) and carbon dioxide evolution rate (CER) for a batch cultivation of *K. marxianus*. While the OUR, CER and heat flow measurements fingerprint the instantaneous metabolic activity of the organism under study, the dielectric spectroscopy data provides an estimation of the total viable cell volume based on capacitance measurements. While CER, OUR and heat flow measurements offer the same type of information about the metabolic state and activity of the cells, they display fundamental differences in terms of robustness, reliability and sensitivity. Furthermore, the OUR profile in this particular case differ significantly from the CER and heat profiles. The sensitivity and robustness of exhaust-gas based metabolic profiling relies, as does every monitoring technique, on the availability of reliable raw data. However, the gas analyser used in the scope of this study has a limited sensitivity and provides inconsistent data for oxygen concentration measurements throughout the culture. Fluctuations due to noise disturbances can be observed in capacitance and heat flow measurements, highlighting the importance of suitable data pre-treatment and processing. Furthermore, the dielectric spectroscopy data refers to a correlation between the capacitance measurements and any suitable off-line determination of biomass to provide an estimation of the total viable cell volume. The instantaneous and universal nature of the heat flow measurements suggest that biocalorimetry is the most promising and reliable of the techniques studied for the monitoring of the process under study.

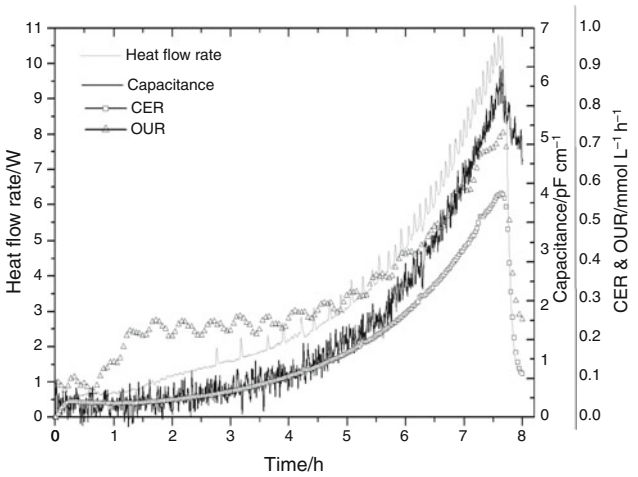

Fig. 2 Comparative profiles of heat flow rate, capacitance, oxygen up-take rate (OUR) and carbon dioxide evolution rate (CER) for a batch cultivation of *K. marxianus* in BioRC1

Correlation studies

Now that the use of calorimetry as an appropriate process analyser has been qualitatively evaluated and justified, correlations need to be drawn between the measured heat flow rate and biomass concentration determined off-line to check the linearity of relationship between the two variables. Biomass profiles based on DCW measurements and heat flow rate exhibit similar behaviour proving the significance of employing the heat flow signal as a key-parameter for monitoring the system under investigation. These profiles also enabled the calculation of the experimental heat yield for the different organisms cultured throughout this study. Table 3 summarizes the average heat yield coefficient value for each yeast strain and allows the comparison with experimental heat yield coefficient from literature. As expected in absence of any additional carbon sources, linear correlations (R^2 above 0.98 for all studies) are observed. The experimentally determined heat yield values were used for the estimation of the biomass concentration and the specific growth rate as described in Eq. 2.

Reliability assessment of the biomass estimator

In order to be able to assess the quality of the developed estimator, it was important to find an appropriate, well-established alternative biomass measurement technique to confirm the values obtained through the heat flow rate. The heat-based model, as described in "Experimental", represents the biomass concentration and is therefore expressed in units of grams of biomass per litre of culture broth. In order to facilitate comparison, validation technique should represent biomass in the same units. Even though dry cell weight (DCW) measurements are prone to gross errors and poor sensitivity, their validity as an off-line validation method is commonly admitted [23–27]. Table 4 summarizes correlation plots between DCW and the heat flow-based biomass estimation for each strain under study. The correlation between DCW and heat-based biomass estimation is between 0.6 and 1.2 for the three strains under study. A one-to-one correlation factor is an indication that the heat-based model is actually able to predict, estimate or measure the biomass determined by DCW in a realistic and tangible way. The accuracy of the estimation is difficult to assess since the biomass measurement based on DCW are not always very accurate themselves and inherently overestimate the actual biomass concentration through accumulation of cell debris over time. Moreover, the DCW estimates the whole amount of biomass in the bioreactor where else the heat flow-based model estimates the viable, metabolically active biomass. Therefore, it could be assumed that DCW measurements systematically overestimate the real biomass concentration which would be reflected by a correlation factor smaller than one between these measurements and the heat-based values. The overestimation of the real biomass concentration through the off-line values compared to the measured metabolic activity can be observed in two out of three cases, namely for *P. pastoris* and *C. utilis*. However, a slight overestimation of the biomass based on heat flow measurements can systematically be observed for *K. marxianus*. It is unclear for the moment if the unexpected overestimation for this strain has a biological meaning. Nevertheless, microscopic observations have shown that the strain tends to form hyphae throughout the culture. The inherent dimorphism of *K. marxianus* could be part of a plausible explanation, but further studies may need to be conducted to determine the validity of this hypothesis. Alternative explanations might be based on finding regarding internal storage materials [28].

In an additional step, the heat-based biomass estimator was tested on-line in batch cultures for each yeast strain. The evolution of the biomass concentration in the batch phase of cultures of *C. utilis, K. marxianus and P. pastoris* is shown in Fig. 3. As expected, according to correlation results shown in Table 4, the biomass estimator shows a certain ability to depict the actual biomass concentration evaluation at least at some stages of the process. Particular attention has to be paid to the smoothness of the heat-based biomass values. In fact, during the batch phase, the only disturbance to the system is the occasional base addition and the sampling. Therefore, the heat flow signal is relatively stable, leading also to a smooth biomass concentration measurement. The developed biomass estimator based on heat flow measurements is reliable and robust as long as the disturbances applied to the system are kept to a minimum.

Table 3 Summary of experimentally determined and reported heat yield coefficients for three different Crabtree-negative yeast strains on two different C-sources

Strain	C-source	Average experimental heat yield ($Y_{Q/X}$)/kJ g^{-1}	Heat yield reported in literature [22, 31] ($Y_{Q/X}$)/kJ g^{-1}
K. marxianus	Glucose	16.8	19.96
C. utilis	Glucose	5.8	11.66
P. pastoris	Glycerol	14.6	11.73

Table 4 Summary of the correlation factors between the estimated biomass concentration based on the metabolic heat flow measurements and the off-line determined biomass based on dry cell weight for the three different yeast strains under study

Strain	Correlation factor (*Y*/*X*)	R^2
K. marxianus	1.21	0.99
C. utilis	0.94	0.97
P. pastoris	0.60	0.97

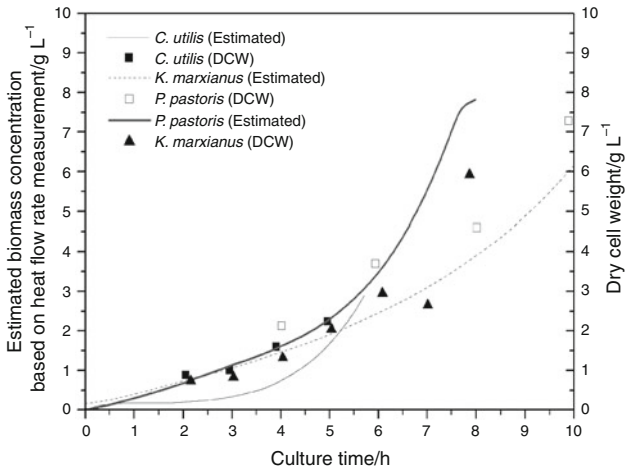

Fig. 3 Comparative profiles of biomass concentration as a function of time for *C. utilils*, *K. marxianus* and *P. pastoris*; the lines (*thick black line* for *C. utilis*, *thin black line* for *P. pastoris*, *dotted line* for *K. marxianus*), represent the on-line estimated biomass concentrations based on the measurements of the metabolic heat flow while the discrete points (*open squares* for *C. utilis*, *filled squares* for *P. pastoris* and *triangles* for *K. marxianus*) represent the off-line determination of the biomass concentration

Specific growth rate estimator and controller robustness

The μ estimator is, according to Equation, derived from the biomass estimator based on calorimetric measurements. To assess the performance of the developed estimator, a fed-batch was carried out, controlling the specific growth rate to a desired set-point (0.2 h^{-1}). The average-specific growth rate based on heat was calculated (0.21 h^{-1}) and the mean tracking error evaluated (0.03 h^{-1}) in order to assess the robustness of the specific growth estimator. In order to determine the long-term stability of the specific growth rate estimator as well as of the controller, the set-point was maintained for over 5 h. The off-line estimation of μ, based on the application of the Monod model for cell growth involving the biomass concentration obtained from DCW measurements, showed concordance with the heat-based values. However, Fig. 4 indicates that the response (black line) is noisy, giving evidence for the need for additional stabilisation of the controller through mathematical techniques in order to create an estimator with a

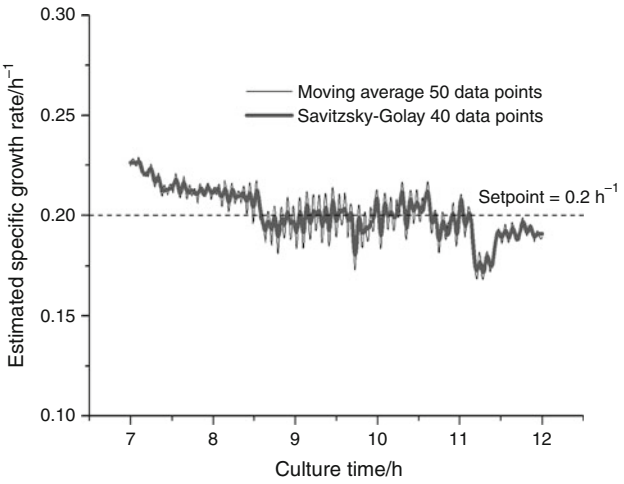

Fig. 4 Evolution of the specific growth rate profile based on the developed estimator throughout a fed-batch culture of *K. marxianus*, where the *black line* represents the original data and the *grey line* data after application of a Savitzky-Golay filter

high potential as a robust control platform for microbial fed-batch cultures. Even though more appropriate techniques may be available, the simple application of a Savitzky-Golay filter over the heat flow measurements showed to improve the stability of the specific growth rate estimator, highlighting the importance of mathematical treatment for enhanced data robustness.

Assessment of broader applicability

The results in section "Reliability assessment of the biomass estimator" highlighted that the developed biomass estimator can be applied to different Crabtree-negative yeasts. It is estimated that little modification of the method for biomass estimation and filtering is required to enable application to other organisms as long as they do not suffer any overflow metabolism leading to a fermentative metabolism. This transferability of the developed model allows a fast development of monitoring and control strategies for different type of organisms since the only important parameter to be known prior a first run is the appropriate heat yield coefficient of the system. This value can be obtained from the literature for several of the most commonly used organisms growing on different C-sources [22] or calculated theoretically based on the molecular composition of the biomass of the strain involved [29]. Furthermore, after an initial run, the heat yield coefficient can be experimentally determined for the given culture conditions. The developed biomass estimator is thereby a simple and flexible tool for in-line monitoring of Crabtree-negative suspension cultures and a firm foundation for the creation of control strategies for fed-batch cultivation.

Conclusions

The implementation of a simple, heat-based biomass and specific growth rate estimator for yeast cell cultures in a bench-scale calorimeter has been successfully demonstrated. The continuous in-line estimation of the biomass gives a real-time insight into the on-going process and serves as a starting point for the development of simple, but flexible control strategies for setting the specific growth rate at a desired level in fed-batch cultures of Crabtree-negative yeast cells. The potential of the developed biomass estimator as a live window into the bioprocess has its importance in the PAT framework as it highlight the potential of biocalorimetry as a non-invasive PAT process analyser and as a firm basis for the development of a PAT platform for monitoring and control.

A PAT platform for monitoring and control of microbial fermentations can be broken down into several components, namely the process parameters and their monitoring, the monitoring tools, the control strategy and the response system.

This study shows that a reliable and robust estimation of process parameters such as biomass and specific growth rate is possible within the limits stated by this report. As for the monitoring tool and the control strategy, this study highlights the potential of biocalorimetry as a PAT tool to successful and controlled microbial fermentations. Indeed, the developed biomass estimator based on heat flow measurements proves to be efficient in monitoring the growth of Crabtree-negative micro-organisms such as *K. marxianus*, *C. utilis* or *P. pastoris*. However, only through the use of carefully chosen averaging and modelling techniques, it becomes feasible to apply this biomass estimator as well as the derived growth rate estimator, as a basis for a control strategy in order to maintain a given specific growth rate for a Crabtree-negative yeast. The investigations are in an early stage of development, but the high potential for industrial application of this calorimetry-based μ-estimator should be highlighted. Indeed, even though a calorimeter, as presented in this article, could be considered as a sophisticated and costly process analyser, gathering the necessary on-line heat flow measurements from an industrial bioreactor in order to infer the rate of heat production requires only minimal changes and the addition of some common instrumentation such as temperature probes, calibration heaters and flow meters. The transformation of an industrial bioreactor into a tool enabling the measurement of heat flow has indeed already been achieved as reported by several authors [14, 30].

The most important part in terms of components of a PAT platform, however, is the response system. Industrial bioprocesses for the development of pharmaceuticals or food products involve for instance organisms such as *Escherichia coli* or *Saccharomyces cerevisiae,* displaying a more complex metabolic behaviour than the microbes under study. Issues related to metabolic activity of Crabtree-positive organisms render a direct correlation between heat production and biomass evolution, as presented in this article, more complex. However, these challenges could be addressed by the use of a second monitoring tool allowing the measurement of the biomass or the metabolic state of the cellular system.

This study underlines the importance of a thought through choice of the model organism in order to minimize difficulties inherent to the response system and their impact onto the monitoring and control platform. Indeed, it was an interesting choice to use Crabtree-negative yeast cells in the first instances in order to avoid dealing with both respiratory and fermentative metabolism. However, it was not foreseen that other characteristics, such as the dimorphism of *K. marxianus* could also influence the outcome of the study.

The aim of subsequent study is the improvement of the reliability and the robustness of the biomass and growth rate estimation based on heat in order to achieve at a later stage close control of the latter. This objective is pursued in two distinctive ways at the moment by developing on one hand an artificial neural network model for biomass estimation through heat measurements and on the other hand by creating an energy-balance based on-line data reconciliation system. Both approaches have their validity, advantages, as well as drawbacks and will be tested and compared in order to achieve the most consistent possible biomass and specific growth rate estimator which would enable the creation of a PAT biocalorimetry platform for highly controlled fed-batch cultures of Crabtree-negative yeasts.

Acknowledgements The Science Foundation of Ireland (SFI—Research Programme Number 08/IN.1/B1948) as well as the Irish Council for Scientific and Engineering Technology (IRCSET—Post Doctoral Fellowship D/2008/44) are greatly acknowledged for financial support of this work. The authors thank Brian Freeland for his considerable assistance in installing the experimental setup.

Appendix

Metabolic heat flow model

Precise measurements of the heat generated by the metabolic activity of living cells are a prerequisite for reliable bioprocess control based on calorimetry. A heat balance over the BioRC1 setup paved the way for an accurate determination [13] of the metabolic heat generation rate and can be stated as follows:

$$\text{Heat inflow} = \text{heat accumulation} + \text{heat outflow} \quad (3)$$

Equation 3 can be written as well as:

$$m \cdot C_{p,r} \frac{dT_r}{dt} = q_r - q_f + q_c + q_s - q_g + q_d - q_l \quad (4)$$

For isothermal conditions as those encountered during fermentations, the heat accumulation stated in Eq. 4 equals zero, leading thereby to Eq. 5.

$$q_r + q_{cal} + q_s = q_f + q_g + q_d + q_l \quad (5)$$

Equation 5 displays the details of the heat flow balance over the reactor, where q_r represents the heat generated by the metabolic activity of the cells, q_c the calibration power, q_s the energy input due to the stirring, q_f the heat flow through the reactor wall, q_g the heat flow caused by the aeration, q_d the heat input due to dosing and q_l the heat flow through the reactor head assembly by radiation and conduction. Equation 5 can be equalled to the variation of the total heat capacity of the reactor as a function of time, leading thereby to Eq. 6:

$$m\, C_{p,r} \frac{dTr}{dt} = q_r - q_f + q_c + q_s - q_g + q_d - q_l \quad (6)$$

For constant operating conditions, q_s and q_g are observed to be constant. Moreover, q_c, the calibration power used for the specific heat transfer coefficient determination is kept at constant at 9 W (standard calibration heater, Mettler Toledo, Greifensee, Switzerland). The heat flow through the reactor wall into the jacket fluid q_f is determined in-line by the WINRC software as stated in "Experimental" during the calibration step preceding each experiment.

$$q_f = U \cdot A \cdot (T_r - T_j) \quad (7)$$

The three parameters, q_s, q_g and q_c are assumed to be constant [13, 15] throughout a given fed-batch experiment, hence, they may be grouped and represented by q_b, the baseline heat flow, which is measured in terms of q_f at steady state operating conditions before the inoculation at each fed-batch run.

Liquid additions in terms of base consumption, as well as substrate consumption during feed phase need to be accounted in the overall heat balance since they are contributing to disturbances in the measured heat flow signal. Heat flow contributions due to liquid additions can be taken in account as given in Eq. 3.

$$q_d = m_{base} \cdot C_{p,base} \cdot (T_r - T_{base}) + m_{substrate} \cdot C_{p,substrate} \cdot (T_r - T_{substrate}) \quad (8)$$

Equation 3 describes the heat term due to liquid additions, where m_{base} and $m_{substrate}$ are the mass flow rate of the base and of the substrate feed solution respectively, $C_{p,base}$ and $C_{p,substrate}$ the specific heat capacity of the base and of the substrate feed solution respectively and T_{base} and $T_{substrate}$ the temperature of the base and of the substrate feed solution. Equation 3 was evaluated in real-time in a LabVIEW program, assuming T_{base} and $T_{substrate}$ to be equal to the ambient temperature (T_a) which is continuously monitored.

Heat loss through the non-thermostated parts of the reaction setup is depending both on the surrounding temperature T_a and the heat transfer coefficients of the different set-up parts. A proportional model for the estimation of q_l was developed based on measurements of the ambient temperature as described in Eq. 9.

$$q_l = \alpha \cdot (T_r - T_a) \quad (9)$$

The previous equations are brought together and result in Eq. 10 describing the metabolic heat flow rate. The q_r values were estimated in LabVIEW and averaged over five points to decrease the influence of the short-term noise on the heat flow signal. The metabolic heat flow measurements serves as a basis to the elaboration of a heat-based biomass and specific growth rate estimator.

$$q_r = q_f + q_d - q_b + q_l \quad (10)$$

Biomass and specific growth rate estimator

Specific growth rate estimators are commonly based on Monod-derived models, requiring the reliable direct biomass measurements such as capacitance measurements [20] or indirect estimations based on oxygen up-take rate [14] for example. However, a heat-yield based approach may be interesting as stated by Duboc and von Stockar [15].

$$Y_{Q/i} = \left| \frac{q_r}{r_i} \right| \quad (11)$$

In the specific case of biomass production rate, the relationship between the metabolic heat flow rate and the heat yield can be expressed as the ration of heat release and the biomass production rate:

$$Y_{Q/x} = \left| \frac{q_r}{r_x} \right| \quad (12)$$

Since the heat release rate is a parameter that is monitored on-line as described by Eq. 12 the biomass production rate can be represented [20] by the following equation:

Combing and rearranging Eqs. 12 and 11 leads to Eq. 13, describing the biomass production rate in gram of cells per unit time. This equation can be integrated (Eq. 15) leading to Eq. 16

$$r_x = \frac{dx}{dt} = x \cdot \mu \quad (13)$$

The solution of Eq. 16 can substitute the term for biomass in Eq. 13 leading to a new form of:

$$dx = \frac{q_r}{Y_{Q/x}} dt \tag{14}$$

$$\int_{x_0}^{x_t} dx = \int_0^t \frac{q_r}{Y_{Q/x}} dt \tag{15}$$

$$x_t = x_0 + \frac{Q_t}{Y_{Q/x}} \tag{16}$$

The solution of the integration displayed in Eq. 15 is shown in Eq. 16, leading to an estimation of the biomass at a given time t as a function of the cumulative heat release Q_t and the heat yield coefficient.

$$Y_{Q/x} = \left(q_r \Big/ \mu \cdot \left(x_0 + Q_t \Big/ Y_{Q/x} \right) \right) \tag{17}$$

Equation 18 permits to express the specific growth rate as a function of the heat production rate q_r, the cumulative heat release Q_t at a given time t and the heat yield coefficient.

$$\mu_{est,t} = \frac{q_r}{Y_{Q/x} \cdot x_0 + Q_t} \tag{18}$$

Equation 18 gives an instantaneous estimation of the specific growth rate at a given time t as long as the reaction volume does not change. Since the different experiments in this study were carried out in fed-batch mode, the changes in volume during the reaction must be taken in account. When accounting for the changes in reaction volume, Eq. 14 is modified as follows:

$$d(x \cdot V_r) = \frac{q_r}{Y_{Q/x}} dt \tag{19}$$

Equation 19, giving the relationship between the heat yield coefficient, the heat release rate and the variation of biomass and reaction volume, might be again integrated as shown in Eq. 20 leading to Eq. 21 which gives an instantaneous estimation of the specific growth rate at a given time t. The solution of can substitute the term for biomass. By modifying the equation, the specific growth rate might be estimated as shown in Equation, relating the heat production rate q_r, the cumulative heat release Q_t at a given time t and the heat yield coefficient.

$$\int_{x_0 \cdot V_0}^{x_t \cdot V_t} d(x \cdot V_r) = \int_0^t \frac{q_r}{Y_{Q/x}} dt \tag{20}$$

$$x_t \cdot V_t = x_0 \cdot V_0 + \frac{Q_t - Q_0}{Y_{Q/x}} \tag{21}$$

The solution of the integration displayed in Eq.20 leads to an estimation of the biomass at a given time t as a function of the cumulative heat release Q_t and the heat yield coefficient.

$$\mu_{est,t} = \frac{q_r}{x_0 \cdot V_0 \cdot Y_{Q/x} + (Q_t - Q_0)} \tag{22}$$

Equation 22 describes the estimation of the specific growth rate as a function of the initial biomass concentration, the heat yield coefficient, the cumulative heat production and the heat production rate.

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