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Quantitative calorimetric investigation of fed-batch cultures of *Bacillus sphaericus* 1593M

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Abstract

For many years, calorimetry has been recognized as a powerful and universal tool for monitoring chemical and biological processes. A laboratory-scale reaction calorimeter (RC1, Mettler–Toledo), initially developed for chemical reaction studies with a sensitivity of 100–150 mW/l, has been improved to enable the monitoring of very low heat production rates (<10 mW/l). A major limitation to successful process control, has been the inability to achieve real-time quantitative calorimetry. This is in part due to the operating principle of the RC1, in which the measured heat signal is calculated from the temperature difference between the reaction mass and the jacket oil and the heat transfer coefficient (*UA*). The latter frequently varies during a reaction, particularly a bioreaction, due to changes in volume, viscosity and cell density, and is difficult to determine accurately during the process.

In the present study, this problem has been solved by a technical modification to the reactor vessel of the RC1. This involves forcing the heat transfer to occur through a well defined and constant area through the creation of a large resistance to heat transfer in the upper part of the reactor vessel. This was achieved by creating an air gap between the reactor contents and the reactor wall through the insertion of a PTFE sleeve. Control experiments undertaken with this modified system, in the absence of any reaction, showed that *UA* remained constant for volume changes as large as 50% of the working volume. Similarly, a simulated fed-batch experiment with monitoring of the stirring power, showed that the baseline heat signal could be accurately and quantitatively corrected for large dynamic variations of the volume.

Using monitoring of the oxygen uptake rate as a reference, this modified system was validated by application to fed-batch cultures of *Bacillus sphaericus* 1593M. This strictly aerobic bacterium produces parasporal insecticidal crystal proteins which are toxic to mosquito larvae. In these fed-batch cultures, the nutrient feed was controlled using measurement of the metabolic heat release, since the latter is proportional to the substrate uptake rates for a given metabolic state. A culture, composed of two repetitive fed-batch cycles followed by a batch cycle, demonstrated that real-time and quantitative signals could be obtained, even for highly dynamic processes in which the volume and agitation rate may vary significantly and where quick repetitive inoculations can be made. The result of this work is a modified RC1 (or Bio-RC1) which is as easy to use as any conventional bioreactor yet has the unique feature of being able to provide an accurate measurement of the energy dissipated as heat in chemical or biological processes, over a wide range of operating conditions.

Keywords: Bioinsecticide; Calorimetry; Bioprocess monitoring; Bioprocess control

Abbreviations: FB*i*, fed-batch culture number *i*; Min, mineral salts concentrated solution; NB, nutrient broth solution; RC1, reaction calorimeter from Mettler–Toledo; Vit, vitamin concentrated solution

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Nomenclature

A	acetate anion concentration (g/l)
c_H	mole of proton per kg of acid solution
C_p	heat capacity (J/g °C)
F	volumetric flow (1°/min), 1°: volume at standard conditions (0 °C and 1 atm)
G	glutamate anion concentration (g/l)
$\Delta H_r(x)$	enthalpy of reaction of equation x (kJ/mol)
$\Delta H_d(x)$	enthalpy of dilution of solution x (kJ/mol)
K	lumped heat transfer coefficient (W/°C)
m	mass (g)
$\dot{m}$	mass flow (g/s)
Mt	torque (Nm)
$\dot{n}$	molar flow (mol/s)
N	ammonium ion concentration (g/l)
p_{O_2}	dissolved oxygen concentration (%)
q	heat flow (W)
r	conversion rate (C mol/h)
RH	relative humidity (%)
S	stirring speed (rpm)
T	temperature (°C)
ΔT	temperature difference between bioreactor bulk and jacket (°C)
UA	overall heat transfer coefficient (W/°C)
V	volume (l)
X	biomass concentration (g/l)

Subscripts

a	acid
ac	accumulation
bc	bubble column
bl	baseline
c	calibration
e	environment
f	feed
g	gas
h	headplate
i	inlet
j	jacket
o	outlet
p	process
r	bioreactor
s	stirring
S	substrate

1. Introduction

Heat exchange is a universal feature of any chemical, biochemical or biological system. Even though it is clearly non-specific, quantitative calorimetry can still yield relevant information about any on-going process when correlated to other variables by appropriate models. An extensive introduction to quantitative biocalorimetry has recently been published [1]. Two main types of calorimeter systems have been intensively developed during the last decades: (1) microcalorimeters [2,3] and (2) lab-scale calorimeters [4]. As the latter are also high performing (bio)reactors, their potential and interest as tools for quantitative monitoring and control of (bio)processes has been well established [5,6]. There are a number of reports on the use of on-line calorimetry to control fed-batch processes. However, these involved only simple on/off control due to the non-quantitative nature of the heat signal [6,7]. The untreated, unfiltered calorimeter signal from an RC1 has been used, in combination with the carbon dioxide production rate, to maintain yeasts in the oxidative metabolic regime, by controlling the feeding of nutrients [8]. The on-line heat signal from a Bio-RC1 (an improved version of the commercial RC1 for biological applications [4]) has also been used to control dilution rate shifts during continuous cultivation of yeasts [6]. In this work, the heat signal was quantitative since there were no changes in the reaction volume or stirring power.

Until now it has been very difficult to obtain quantitative calorimetric information from fed-batch culture systems since the volume changes continuously in a non-linear fashion and high cell densities are attained, which requires variation of the agitation rate to enhance oxygen transfer. These variations affect the hydrodynamics of the reactor mass and induce changes in the overall heat transfer coefficient and stirring power input. While the latter can be accurately measured on-line using a torque meter or electrical wattmeter [9,10], there is no way to directly monitor changes in the overall heat transfer coefficient. The present work describes the development and experimental validation of a new vessel design for the Bio-RC1 which maintains a constant heat transfer coefficient over a very wide range of culture conditions.

In order to test this new system, cultures of the aerobic, spore-forming bacterium *Bacillus sphaericus*

have been undertaken. This organism was chosen since it represents a number of challenges for quantitative calorimetry: (1) it grows aerobically, and therefore, requires aeration which results in a number of heat flows; (2) the morphology of the bacterium changes during the culture due to sporulation and the excretion of proteases which results in foaming and variations in the stirring power [10]; (3) the bacterium has potential commercial interest since it produces proteins which are toxic to mosquito larvae of *Culex*, *Anopheles* and *Aedes* species, organisms which transmit filariasis, malaria and yellow fever, respectively [11,12]. In a previous work, it was shown that growth of *B. sphaericus* is inhibited by acetate. Thus, feeding of a culture by the addition of discrete quantities of substrates which had become depleted was not optimal. This was due to the growth rate drastically decreasing after depletion of a substrate and which lead to a rapid and irreversible initiation of sporulation. This work will demonstrate that quantitative calorimetry can be used to feed substrates at the rate at which they are consumed thereby enabling cells to grow at the maximum rate due to non-limiting and non-inhibiting substrate concentrations.

2. Theory

The heat flows around the Bio-RC1 used in this work are shown in Fig. 1. Heat flows are defined as positive if heat is released within the bioreactor. For an aerobic bioprocess, the heat balance around the reactor can be written as follows:

$$q_{ac} = q_p - q_j + q_s - q_g - q_e - q_f - q_a - q_{CO_2} \quad (W) \tag{1}$$

where q_{ac} is the heat accumulation in the reactor mass, q_p the heat flow of the running process, q_j the heat flow through the reactor wall to the jacket oil, q_s the stirring power, q_g the heat flow induced by aeration, q_e the heat flow to the environment through the non-jacketed part of the reactor, q_f the heat flow of the feed, q_a the heat flow of the acid or base addition and q_{CO_2} the heat flow of CO_2 vaporization.

For bioprocesses with a good bioreactor temperature controller, the heat accumulation term may be neglected. The jacket heat flow is the most important

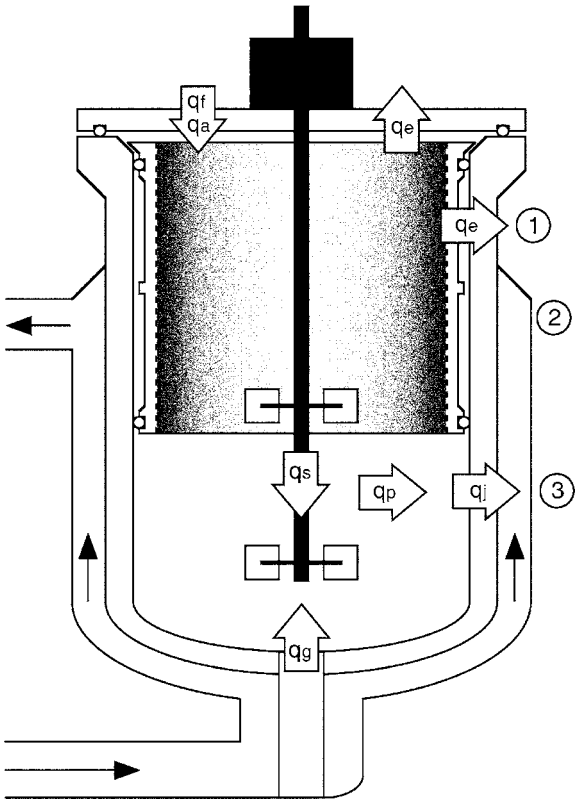

Fig. 1. Schematic representation of heat flows around the Bio-RC1 with the insulating PTFE cylinder inserted in the glass reactor. Numbers and terms are explained in the text.

to monitor since it represents the sum of all other heat flows, including the heat flow generated by the (bio)process. In isothermal mode, T_j is varied by the temperature controller to maintain the bioreactor temperature constant. In the case of the RC1, q_j is given by Eq. (2):

$$q_j = UA(T_r - T_j) = UA\,\Delta T \quad (W) \tag{2}$$

When all the other heat flows are constant, the heat transfer coefficient can be determined with a known or measured calibration heat flow (q_c). In the case of the Bio-RC1, this calibration is made using an electrical heater releasing a measured quantity of heat.

The heat flow produced by stirring can be important, compared with the process heat flow, and in most calorimetric systems is usually not directly measured. In this work, a torque meter was used to measure q_s,

and the heat flow calculated using Eq. (3):

$$q_s = 2\pi \frac{S}{60} \mathrm{Mt} \quad (\mathrm{W}) \tag{3}$$

The heat flow due to aeration, q_g, is a complex function which depends on a number of parameters described in Eq. (4) [13]. In this work, this term has not been measured but evaluated calorimetrically:

$$q_g = f(T_{gi}, T_{go}, \mathrm{RH_i}, \mathrm{RH_o}, \dot{m}_{gi}) \quad (\mathrm{W}) \tag{4}$$

The heat losses to the environment, q_e, can be calculated from a single relation (Eq. (5)), in which one lumped heat transfer coefficient, K_e, for the upper part of the bioreactor is used. The term q_e only has a significance when important fluctuations in the ambient temperature occur:

$$q_e = K_e(T_r - T_e) \quad (\mathrm{W}) \tag{5}$$

Any mass added to the bioreactor will also induce a heat flow. The heat flow for substrate feed (Eq. (6)) and acid (Eq. (8)) are described. The substrate feed solution can be considered to be an ideal solution in which the heat flow arises from the temperature difference between bioreactor contents and the feed solution. For acid addition, the concentrated acid solution is not ideal and will provoke heat effects when diluted. Protons added will be neutralized and generate heat (Eq. (7)). For sulfuric acid concentrations (2 and 4 mol/kg of solution), the heat of dilution, ΔH_d(a) was estimated to −4 kJ/mol of proton [14,15]:

$$q_f = \dot{m}_f C_{p_f}(T_r - T_f) \quad (\mathrm{W}) \tag{6}$$

$$\mathrm{H^+ + OH^- \rightleftharpoons H_2O}, \quad \Delta H_r = -55.9\,\mathrm{kJ/mol} \tag{7}$$

$$q_a = \dot{m}_a(C_{p_a}(T_r - T_a) + c_H \Delta H_r(7) + c_H \Delta H_d(a)) \quad (\mathrm{W}) \tag{8}$$

During the bioprocess, carbon dioxide is produced. Since the pH is controlled, CO_2 is continuously stripped by the aeration and accumulation in the medium may be neglected. The resulting heat flow term is then only a function of the stripping enthalpy (Eq. (9)) [15]:

$$\mathrm{CO_2(aq) \rightleftharpoons CO_2(g)}, \quad \Delta H_r = +20.3\,\mathrm{kJ/mol} \tag{9}$$

$$q_{CO_2} = \dot{n}_{CO_2} \Delta H_r(9) \quad (\mathrm{W}) \tag{10}$$

The heat flow of an on-going (bio)process, q_p, is the result of the energy dissipated by all (bio)chemical reactions involved. As these cannot be directly measured, it can only be determined from the following relation (Eq. (11)):

$$q_p = q_j - q_s - q_c + q_g + q_e + q_f + q_a + q_{CO_2} \tag{11}$$

When there is no (bio)chemical activity in the reactor, the sum of all the heat flows on the right side of Eq. (11) should be zero, providing that they have been correctly measured and calibrated. In practice, Eq. (11) may be simplified by lumping all the constant heat flow terms in a "baseline" term, q_{bl}, which has to be determined before and after any experiment. If culture conditions are not varied, q_s, q_g, q_e and q_f are constant. Finally, for monitoring purposes, q_a and q_{CO_2} may be lumped in q_p, since these are related to the metabolic activity of cells. They only need to be considered separately for thermodynamic evaluations of q_p. For the basic (Bio-)RC1 system, Eq. (11) may therefore, be simplified to Eq. (12):

$$q_p = q_j - q_c - q_{bl} \tag{12}$$

For the case of fed-batch cultures, this work will show which heat flow terms may be effectively lumped together in the baseline as well as precautions that have to be taken for the correct determination of q_j. The standard procedures for calibration and evaluation of the RC1 heat signal (q_j) are described in the Section 3.4.

3. Materials and methods

3.1. Bioreactor set-up

The bioreactor used was a RC1 calorimeter (Mettler–Toledo AG, Switzerland) modified for bioprocess operation [4]. An additional electronic interface (Model RD10, Mettler–Toledo AG, Switzerland) was used to read the data from two balances. The temperature of the reactor headplate and nutrient feed heat exchanger were thermostated using a circulating waterbath (Model DC3, Haake, Germany). Air supply was controlled using a thermal mass flowmeter (Model 5850 TR, Brooks, The Netherlands). The filtered air (0.2 μm) was preheated and humidified by passage through a custom built sterilizable and thermoregulated bubble column.

The data acquisition system was composed of a 16 bit A/D board (Model NB-MIO16-XH, National Instruments, USA) coupled to a computer (Model PowerMac 7100/66) operating with LabV/EW 4.0 software (National Instruments, USA). The reactor temperature (T_r), jacket temperature (T_j), calibration power (q_c) and stirring speed (S) data were extracted from the RC1 communication protocol (RS-422) after conversion to RS-232 protocol. The mass of acid (m_a) and nutrient feed (m_f) added were measured by placing reservoirs containing the acid and feed solutions on balances (Model PM2500, Mettler–Toledo AG, Switzerland) connected to the RC1 via the RD10 interface. The balance data were extracted from the RD10 communication protocol in the same way as for the RC1. The data from other process variables were acquired from the 16 bit A/D board: (1) the pH was measured using a pressurizable Ingold electrode (Mettler–Toledo AG, Switzerland) connected to a pH controller (Bioengineering, Wald, Switzerland); (2) the dissolved oxygen concentration (p_{O_2}) was measured using a 12 mm diameter polarographic Ingold electrode (Mettler–Toledo AG, Switzerland) coupled to a custom made amplifier; (3) the ambient (T_e) and headplate temperatures (T_h) were measured using pre-calibrated Pt100 platinum resistance thermometers connected to custom made amplifiers; (4) the stirrer torque (Mt) was measured using a torque meter (transducer TG-02, processing unit AEG 093, Vibrometer Ltd., Switzerland) inserted between the motor and drive shaft; (5) the oxygen (y_{O_2}) and the carbon dioxide (y_{CO_2}) concentrations in the exhaust-gas were measured using paramagnetic (Model 540A, Servomex, England) and infrared analyzers (Model Binos 100, Rose-mount Analytical, USA), respectively. The water vapor pressure in the exhaust-gas sent to the gas analyzers was reduced using a microtube drier (Model PD-750-24SS, Perma Pure Inc., USA). LabVIEW was programmed to read the analog signals and the serial RS-232 ports, to filter voltage data, to calculate real variables via linear calibrations and to send control commands at the frequency of the RC1 communication protocol (2 s). The data were then averaged and plotted over scalable time intervals and stored as tabulated text sheets. The nutrient feed peristaltic pump (Model Alitea, Watson Marlow, UK) was controlled using a voltage input calculated via the calorimetric signal and a series of calibration parameters.

3.2. Biological system

3.2.1. Strain

The bacterial strain *B. sphaericus* 1593M was used for all work. The strain was a kind gift of The Center for Biotechnology, Anna University, Madras, India [16]. Spore suspensions were subjected to a heat shock of 10 min at 80 °C and subsequently stored at −18 °C in 20% glycerol.

3.2.2. Media

Complex medium, nutrient broth, was composed of (g/l): meat peptone 10, yeast extract 5, and NaCl 3. For solid medium, 15 g/l of agar were added to the complex medium. Semi-defined medium was prepared step-wise from concentrated stock solutions of mineral salts (Min), vitamins (Vit) and substrates. All solutions were sterilized through 0.2 μm filters (Acro 50, 0.2 μm, Gelman Inc., USA). All concentrations are given in as initial bulk concentrations. The basic medium was composed of (g/l): $Na_2HPO_4{\cdot}2H_2O$, 2.63; KH_2PO_4, 0.84; $(NH_4)_2SO_4$, 4 and 1% of nutrient broth solution. Mineral salts were prepared as a 200-fold concentrated solution and autoclaved. The salts were mixed in the following order (g/l): nitrilotriacetic acid, 0.076, pH adjusted to 4; $CaCl_2{\cdot}2H_2O$, 0.041; $MnCl_2{\cdot}4H_2O$, 0.016; $FeSO_4{\cdot}7H_2O$, 0.011; $MgSO_4{\cdot}7H_2O$, 0.39; pH readjusted to 4. Vitamins were prepared as a 50-fold concentrated solution (g/l): biotin (+), 0.003; pH adjusted to 6.5; thiamine–HCl, 0.03; pH adjusted to 6.5. Antifoam A (Merck, USA) was added to a final concentration of 0.05% (w/v). Monosodium glutamate$\cdot H_2O$ (2.92 g/l) and sodium acetate$\cdot 3H_2O$ (3.93 g/l) were added to the basic medium as carbon and energy sources.

3.2.3. Culture conditions

For inoculum preparation, stock spore suspensions were plated on nutrient broth agar plates at 30 °C and a single colony used to inoculate 10 ml of the complex or semi-defined medium. After incubation with shaking at 30 °C for 24 h, 2 ml of this cell suspension were used to inoculate a 1 l baffled Erleneyer flask containing 100 ml of medium. After incubation with shaking for 24 h at 30 °C, the content of the flask was centrifuged at 5000 × g for 15 min and the cell pellet re-suspended in, approximately 5 ml of medium. The RC1 calorimeter (2 l total volume) was inoculated with this con-

centrated cell suspension. The initial conditions of the RC1 cultures are reported in the legends of the figures.

3.3. Analytical

3.3.1. Biomass

Biomass concentration was determined spectrophotometrically and as dry weight. For dry weight determination, culture samples were filtered through pre-weighed, dried, cellulose membranes (Supor®200 or Tuffryn®200, 0.2 μm, Gelman, USA) and the retentate washed with water. The filtrate was stored at −20 °C and used for analysis of substrates. All membranes were dried for 15 min in a microwave oven (150 W) and weighed before and after filtration [17]. Optical density was measured spectrophotometrically (U-3210, Hitachi, Tokyo, Japan) at 600 nm against a water blank.

3.3.2. Substrates

Acetate and phosphate concentrations were determined by HPLC analysis with the following method: isocratic elution, column temperature 50 °C, buffer of 0.0025 mM H_2SO_4, pH 5.3, pre-column Supelguard C610 H (Sigma, USA), column Supelcogel H 300 mm × 7.8 mm (Sigma, USA), with refractive index detector.

Glutamate concentration was determined by HPLC analysis based on ion moderated partition with the following characteristics: isocratic elution, column temperature 85 °C, 2 mM $Ca(NO_3)_2$ buffer, pH 6.5 degassed with helium, Carbo-C 30 mm × 4.6 mm pre-column (Bio-Rad, USA), Aminex HPX-87C 300 mm × 7.8 mm column with differential refractive index detector. Ammonium concentration was determined enzymatically using a standard assay system (Roche, Switzerland).

3.4. Calculations

Yields were calculated based on plots of cumulated quantities of variables and linear regression as previously described [1].

3.4.1. Calorimetric evaluation

Process heat flow is determined from Eq. (12) and the following procedure. The Bio-RC1 reactor is prepared for operation under culture conditions and the initial baseline (ΔT_{bl}) allowed to stabilize. The determination of *UA* is made immediately prior to inoculation by use of the in situ calibration heater. Once the culture has been completed, cells are inactivated by addition of 2 mM sodium azide and a second calibration performed, once the baseline has stabilized, to determine the final value of *UA*. Depending upon the operating conditions it is possible that: (1) the initial and final values of *UA* and q_{bl} are constant and as a result the value of q_{bl} does not need to be corrected off-line; (2) the value of *UA* varies during the experiment, while q_{bl} remains constant in which case the value of *UA* during the experiment is assumed to vary linearly and q_p corrected accordingly; (3) *UA* remains constant throughout the experiment whereas the initial and final values of q_{bl} are different in which case the value of q_{bl} during the experiment is assumed to vary linearly and q_p corrected accordingly; (4) *UA* and q_{bl} vary during the experiment in which case both *UA* and q_{bl} are re-calculated as described in points (2) and (3); (5) if culture conditions are modified (aeration rate, stirring speed, volume, etc.), *UA* and q_{bl} will shift at the time of the modification(s). In this case, baseline stabilization and *UA* calibration must be repeated for each set of culture conditions; (6) corrections of q_{bl} for any measured power, such as agitation, gas flow rate, etc. must be made before addressing points (1)–(4).

4. Results and discussion

4.1. Experimental development

In order to modify the Bio-RC1 vessel, a simple device was installed within the reactor in order to decrease the heat transfer surface (*A*) while maintaining it constant irrespective of other process variables. The specific heat transfer coefficient (*U*) is generally constant for operating conditions used in biological processes since the heat resistance in the inner surface of the vessel wall, which is expected to be rather constant, is anyway small compared to the total heat resistance. As a result, the overall heat transfer coefficient (*UA*) will be constant throughout the process [18]. A schematic representation of the modified Bio-RC1 is given in Fig. 1. The main modification, compared with the existing Bio-RC1, is the insertion of a cylinder within the reactor vessel which creates an air gap

between the reactor mass and part of the inner glass wall of the vessel. Since the heat transfer coefficient of air is, approximately 500-fold higher than that of glass, heat only flows through the glass wall which is not covered by the cylinder [19]. A number of construction materials for the cylinder were tested which could withstand repeated sterilization cycles, did not exhibit excessive thermal deformation, yet were sufficiently elastic as to avoid excessive stresses on the glass reactor during heating/cooling cycles. Of these, PTFE was shown to have the optimum properties and was used for all further work. The external diameter of the PTFE cylinder was deliberately chosen to be 10 mm smaller than the inner diameter of the reactor vessel, such that an air gap was created between them. In order to avoid sepage and accumulation of liquid in this air gap, and consequently dead zones and a heat bridge, *o*-ring seals were inserted at both ends of the cylinder together with elastic steel rings over the inner PTFE surface in order to exert a force to maintain a pressure on the *o*-rings, even should the PTFE deform. This system could be replaced in future by the construction of a glass reactor vessel in which a second, glass-walled air jacket is incorporated.

This system has the advantage that, the heat transfer area is small, independent of all other variables, and thereby increases the theoretical resolution of the heat signal which is given by $0.4 \times UA$ (where 0.4 mK is the resolution of the difference between the T_r and T_j sensors [4]). In addition, in the upper part of the reactor (① in Fig. 1) above the jacket, heat losses to the surroundings are reduced to virtually zero, due to the heat transfer resistance being very high while splashing of the reactor mass on the wall is prevented.

In order to test the efficiency of the modifications, a series of experiments were carried out to verify: (1) under steady state conditions, that volume changes had little effect on *UA*, while simultaneously allowing precise calibration of the torque meter to enable on-line correction of the heat signal and (2) the system under dynamic operating conditions, such as those operating under fed-batch culture conditions.

4.2. Effect of volume changes on the heat transfer coefficient

In order to check the effect of volume changes on *U* (*A* being constant due to the PTFE cylinder), an experiment was carried out in which the reactor was filled with 1.5 l of water and between 90 and 100 ml removed at discrete time intervals. Before and after each volume change the heat signal was allowed to stabilize and two to three *UA* calibrations performed. All other variables were kept constant. The process heat flow reported in Fig. 2 was then calculated from Eq. (11), in which q_f, q_a and q_{CO_2} do not appear since there is no reaction occurring and where q_g was considered to be constant for a given aeration rate. Variations of q_e could be neglected since the ambient temperature only varied between 27.5 and 28.2 °C. The results (Fig. 2) show that *UA* (●) is indeed constant (3.44 W/°C) and independent of volume and stirring power, whereas in the absence of the PTFE sleeve (data not shown) *UA* decreased from 10.4 to 9.4 W/°C for a 0.5 l decrease in volume. More impressively still a volume change of 0.84 l, is accompanied by a decrease in the jacket heat flow from 5 to −0.2 W, yet the calculated process heat flow can be maintained at a value of 0 (±25 mW) through correction with the stirring power. As a result, this experiment also allowed calibration of the torque meter signal and the introduction of a correction factor in Eq. (3), which was used for all future experiments (Eq. (13)):

$$q_s = 0.9654\, 2\pi \frac{S}{60} \mathrm{Mt} \quad \text{(W)} \tag{13}$$

In a second experiment, the effect of volume changes was determined by simulating a fed-batch culture by adding a feed of water. In this case, the q_f term is present, and considered to be constant, and as a result, part of the baseline heat signal (Fig. 3). The Bio-RC1 was operated under culture conditions except that the reactor contained an initial volume of 0.7 l water, which was increased to 1.4 l by the continuous addition of water (Fig. 3). Since a real microbial culture would have a nutrient feed which increases exponentially to maintain a constant specific growth rate, the simulation involved an automatic increase in feed rate by 11 g/h after each addition of 10 g of feed. In a dynamic situation, the jacket heat flow (q_j) can be very efficiently corrected for changes in stirring power (q_s), while the calculated process heat flow (q_p) can be kept steady around zero within a range of ±40 mW except at the end of the feed (Fig. 3). During this latter period, the feed rate was

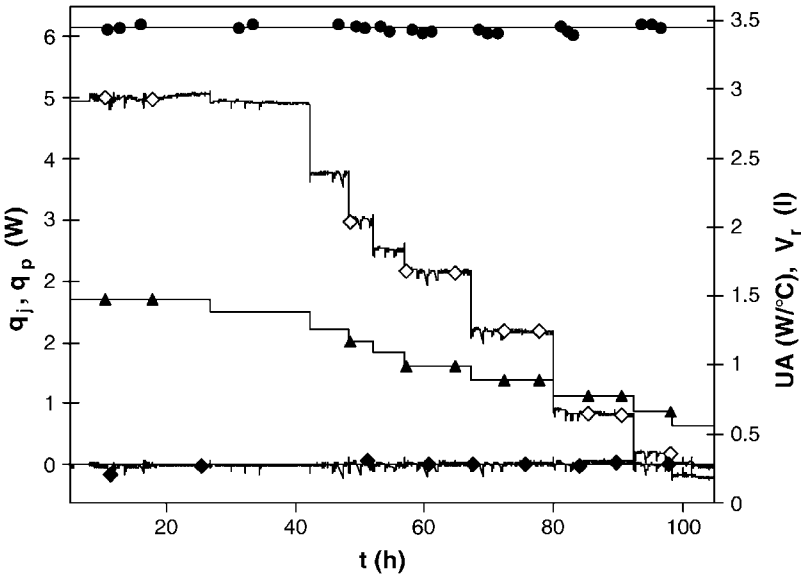

Fig. 2. Effect of volume changes on the heat signal of the Bio-RC1 calorimeter containing the PTFE sleeve: (◇) q_j; (◆) q_p; (▲) V_r; (●) *UA*. Initial conditions: $V_r = 1.48$ l; $F_g = 1.06$ l°/min; $S = 600$ rpm; $T_h = 30.3$ °C; $T_{bc} = 30$ °C. Volume is decreased step-wise down to 0.66 l. All other variables are kept constant.

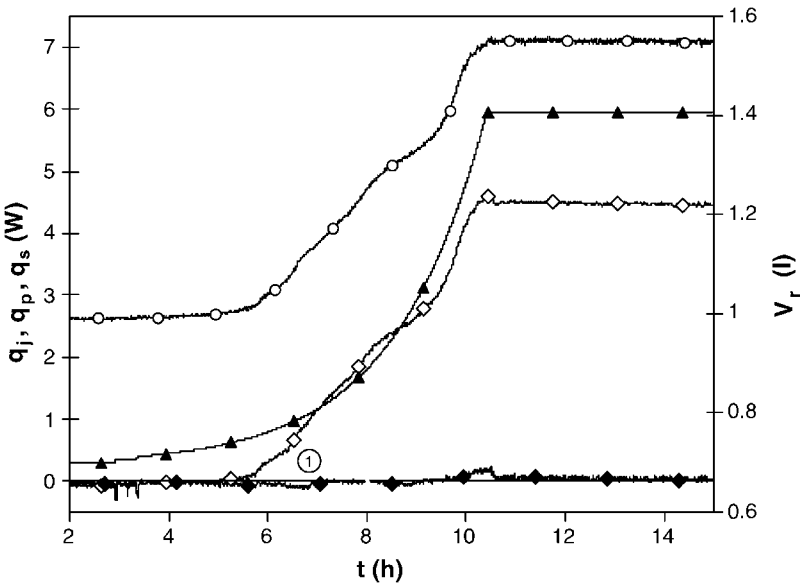

Fig. 3. Effect of blank fed-batch simulation on the heat signal of the Bio-RC1 calorimeter containing the PTFE sleeve: (◇) q_j; (◆) q_p; (○) q_s; (▲) V_r. Initial conditions: $V_r = 0.7$ l; $F_g = 1.81$ l°/min; $S = 600$ rpm; $T_h = 30.3$ °C. Feed rate is incremented based on the feed mass added. F_g and S are kept constant. Numbered events: (①) $T_h \nearrow 30.6$.

very high (>300 g/h) and the stirring power increased very rapidly, resulting in perturbations in the RC1 temperature controller and inducing a small heat accumulation term. During the experiment, the temperature of the headplate and feed thermostating bath was increased to keep the baseline heat signal steady (①, Fig. 3). By setting this temperature (T_h) to 30.6 °C, the heat flow of the feed could be neglected. The two simulation experiments demonstrate that, for important changes of volume or stirring power, the modified Bio-RC1 incorporating a PTFE sleeve, should be able to monitor accurately and quantitatively the heat flow induced by a running (bio)process. In order to test the system under real bioprocess operating conditions, two fed-batch cultures of *B. sphaericus* were performed.

4.3. Efficiency of air pre-treatment

The ability to monitor the stirring power accurately was used to measure the amount of heat taken-up by aeration and to quantify by the efficiency of the bubble column used for air pre-saturation and the thermostating. In this experiment, the Bio-RC1 was operated under culture conditions, except that 1.5 l of water and an aeration rate of 1.06 l°/min were used, and q_g varied. Profiles of q_p, q_j and q_s are reported in Fig. 4. During phase ①, the inlet air stream was passed through the bubble column. After 10 h, the bubble column was by-passed and the inlet air stream connected directly to the bioreactor. During phase ③, aeration was stopped. As expected, the heat transfer coefficient was the same during all three phases, however, q_s varied significantly with aeration rate. When the bubble column is by-passed the calculated q_p, which reflects the heat flow of aeration, an additional 0.27 W is taken-up by aeration, whereas when aeration is stopped, q_p decreases by 0.67 W. As a result, it is clear that the bubble column does not eliminate the heat flow associated with aeration, although in practical terms, it reduces slightly water evaporation from the bioreactor. If it is assumed that air enters the system at 20 °C and leaves at 30 °C, a maximum aeration heat flow of 1.81 W can be calculated, which is much more than the 0.67 W measured. This clearly indicates that the exhaust air of the bioreactor is far from water saturation even though it is intensively mixed.

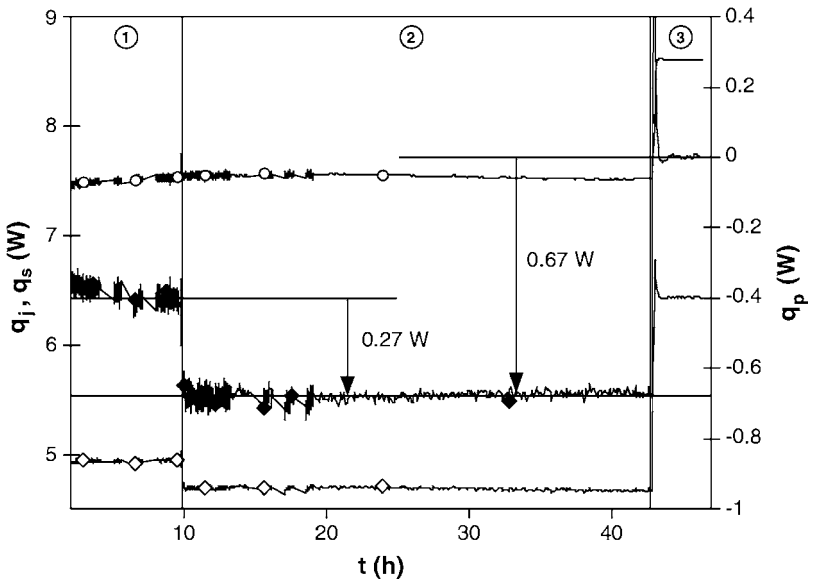

Fig. 4. Determination of the efficiency of an air inlet humidifier through calorimetric measurements: (◇) q_j; (○) q_s; (◆) q_p. Initial conditions: $V_r = 1.48$ l; $F_g = 1.06$ l°/min; $S = 600$ rpm; $T_h = 30.3$ °C; $T_{bc} = 30$ °C. Numbered phases: (①) initial conditions; (②) same conditions but humidifying column is disconnected from the aeration stream; (③) aeration is stopped.

4.4. Repeated fed-batch cultures on acetate and glutamate

Previous experiments showed that the optimal feeding strategy for this bacterium was a feed proportional to the substrate consumption rate, keeping substrate concentrations non-limiting and non-inhibiting. In this work, the substrate consumption rate (r_S) was estimated with the process heat flow and the yield of enthalpy over substrate. This rate was then used to calculate the voltage input to the feed pump knowing the substrate feed concentration and the feed pump calibration.

B. sphaericus was grown in a repeated fed-batch culture in the modified Bio-RC1 (Fig. 5). Cells grew exponentially at a rate of $0.33\,h^{-1}$ for 9.5 h until the process heat flow (q_p) reached a constant value. The nutrient feed was stopped and, after a pulse of mineral salts and vitamins (event ①), growth continued at a lower rate up to 11 h when again q_p became constant. At event ② (Fig. 5), cells were harvested by centrifugation and used to re-inoculate (40 min later) 700 ml of fresh medium and a second fed-batch initiated. During this second fed-batch, cells grew exponentially at a rate of $0.16\,h^{-1}$, for 3.4 h until q_p reached a maximum which was followed by a rapid fall in cell concentration due to sporulation. Pulses of mineral salts, vitamins and nutrient broth solutions at event ③ had no effect on growth. At event ④, cells were harvested once again by centrifugation and used to re-inoculate (40 min later) 700 ml of fresh nutrient broth medium to allow a good sporulation. From the substrate concentration profiles it can be seen (Fig. 5) that the control proportionality was too high during the first fed-batch phase due to incorrect calibration of the nutrient feed pump.

All heat flows measured during this experiment are reported in Fig. 6. When the stirring speed is constant, stirring power and volume profiles are similar, however, the stirring power varied significantly with changes in volume and stirring speed (Fig. 7). The culture volume has been corrected for the volume of samples, pulse additions of nutrients and the stripping rate of water through aeration (9.3 g/h). The

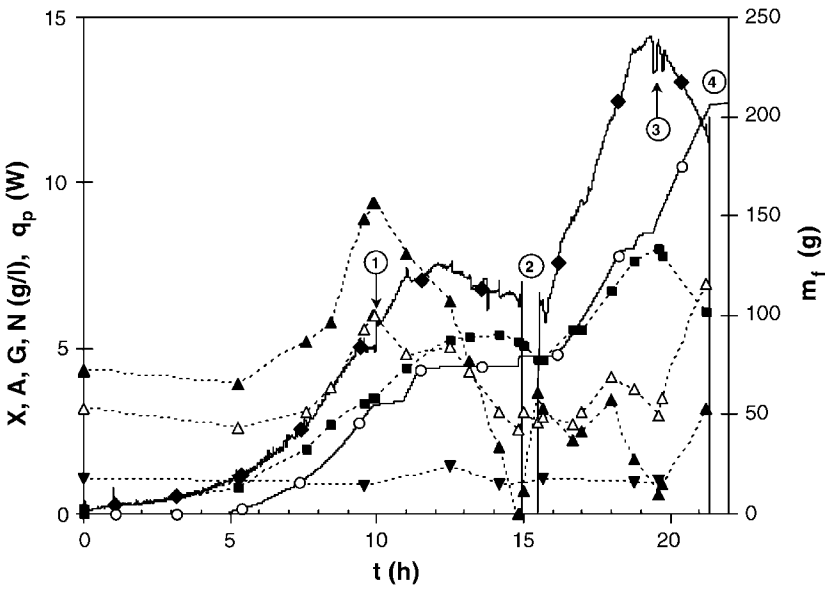

Fig. 5. Substrate and product profiles during a fed-batch culture of *B. sphaericus* on defined medium containing glutamate and acetate. Feeding is proportional to the calorimetric signal: (■) *X*; (▲) *A*; (△) *G*; (▼) *N*; (◆) q_p; (○) m_f. Initial conditions: $V = 0.7$ l; $F_g = 3.21\,l°/min$; $S = 700$ rpm; $T_h = 30.6\,°C$. Numbered events: (①) pulse of 7 ml Min and 7 ml Vit; (②) spin 520 ml of bulk and re-inoculate 0.7 l of identical medium with 46 ml; (③) sequential pulses of 7 ml Min, 7 ml Vit and 7 ml NB; (④) spin 520 ml of bulk and re-inoculate 0.7 l of half concentrated NB with 100 ml.

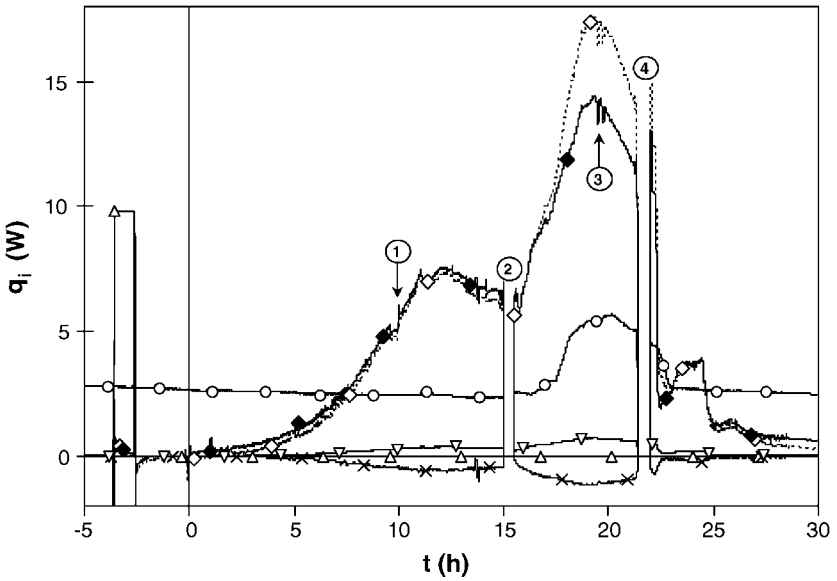

Fig. 6. Heat flows profiles during the same culture as Fig. 5: (◇) q_j; (◆) q_p; (○) q_s; (△) q_c; (×) q_a; (▽) q_{CO_2}. Initial conditions and numbered events found in Fig. 5. From 17 to 20 h, S is smoothly increased up to 895 rpm to maintain p_{O_2} over 20%. From 20 to 23 h, S is smoothly decreased back to 700 rpm.

significant increase of q_s between 17 and 23 h is due to the increase of stirring speed to maintain the dissolved oxygen concentration (p_{O_2}) above 20%. In this culture, the volume reached a minimum of 550 ml after 15.5 h and a maximum of 870 ml after 21.3 h. The q_j was calculated using a value of 3.44 W/°C for the heat transfer coefficient and needed no further correction. The correction of the baseline heat flow

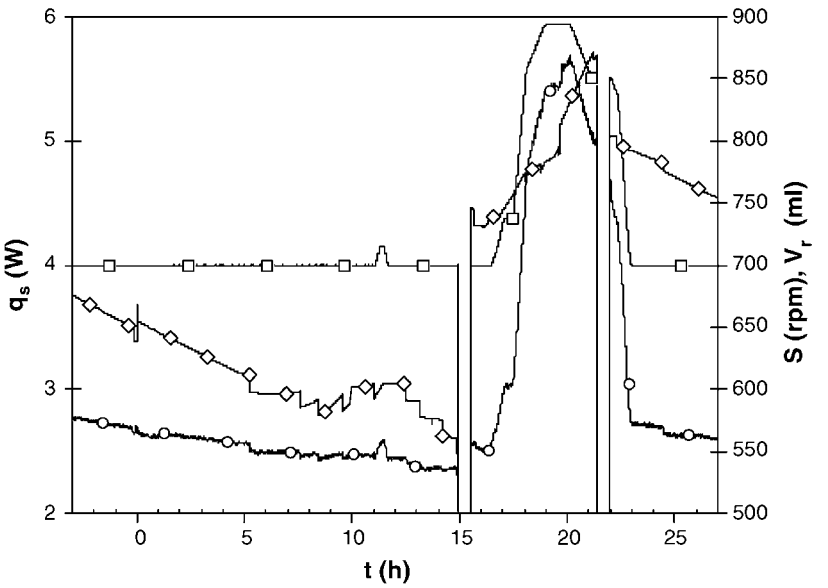

Fig. 7. Stirring power, stirring speed and volume profiles during the repetitive fed-batch of Fig. 5: (○) q_s; (□) S; (◇) V_r.

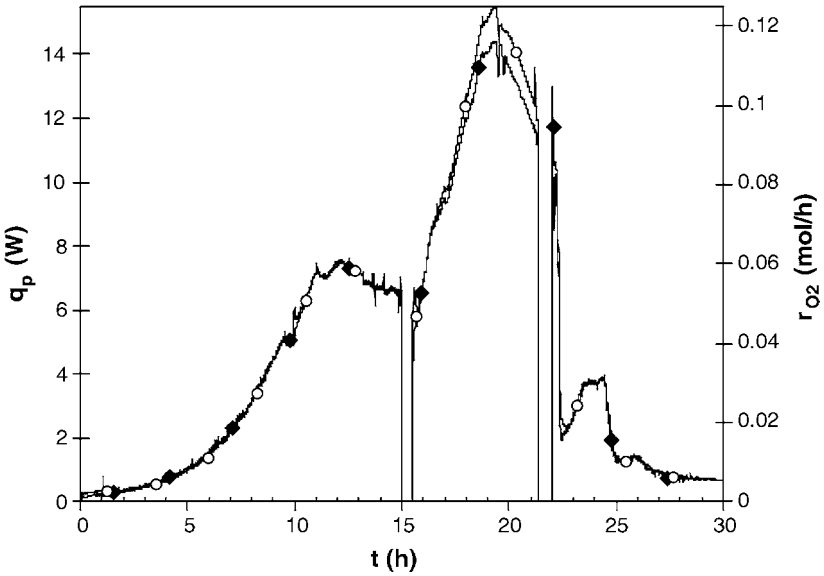

Fig. 8. Process heat flow and oxygen uptake rate comparison during the repetitive fed-batch of Fig. 5: (◆) q_p; (○) r_{O_2}.

using on-line measurements can be seen to function perfectly as shown by the absence of any shift during the electrical calibration performed 4 h prior to inoculation (Fig. 6), and after inoculation. As a result, complete confidence can be given to the calculated values of q_p.

Comparison of the process heat flow (q_p) with the oxygen uptake rate (Fig. 8) shows that in the first fed-batch phase, both rates correlate well (450 kJ/mol of oxygen) with the theoretical value of 460 kJ/mol [6] whereas during the second fed-batch phase, the measured yield is slightly lower (423 kJ/mol). Although the stirring power was much higher during this second phase (Fig. 7), this lower yield cannot be due to an increase of the heat transfer coefficient with the stirring power. It must, therefore, be concluded that this reflects a different metabolic state of the cells. This experiment clearly demonstrates that process heat flow can be monitored quantitatively using the modified Bio-RC1 during repetitive cultures and for a wide range of culture conditions, and thus provides a high degree of operating flexibility. This is mainly the result of the heat transfer coefficient being independent of volume, such that repetitive cultures can be made without the need for regular baseline determinations and *UA* calibrations.

5. Conclusions

Design modifications to the Bio-RC1 vessel resulted in important improvements to the accuracy and operating flexibility of the system. Thus, the overall heat transfer coefficient could be maintained constant over a wide range of operating conditions used in biological processes. In addition, it was shown that it is only necessary to measure the stirring power in order to correct the jacket heat flow to obtain a quantitative and real-time determination of the heat released by a range of bioprocesses. With both the improved vessel design and the stirring power monitoring, the process heat flow could be measured with a high accuracy for dynamic volume changes as large as 0.7 l and for dynamic stirring speed variations resulting in stirring power changes as large as 5 W. This system enables the elimination of the need for frequent *UA* calibrations, and a reduction in baseline shifts due to variations in the aeration rate. These two points improve significantly the operating flexibility of the Bio-RC1 since this allows a significant reduction in preparation time, enables operation of repetitive cultures, and eliminates the need for biomass inactivation at the end of the experiments to make calibrations for off-line baseline correction. The results also show that it is

not necessary to pre-saturate or thermostat the inlet air stream, which results in simplification of the installation. The Bio-RC1 is, therefore, as easy to use as any conventional bioreactor, yet provides real-time quantitative and accurate measurements of the heat associated with a process, allowing it to be used for control purposes. These improvements may equally be applicable to calorimetric investigations of many chemical processes, particularly those inducing a low or constant heat resistance at the inner side of the reactor wall. Processes inducing a high heat resistance may also be quantitatively studied by simply measuring the stirring power with a torque meter, since this resistance is correlated to the thickness of the liquid film at the wall surface which is, in turn, correlated with viscosity.

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