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The application of heat flux measurements to improve the growth of mammalian cells in culture

R.B. Kemp*, Y.H. Guan

Institute of Biological Sciences, Edward Llwyd Building, University of Wales, Aberystwyth, Wales SY23 3DA, UK

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Abstract

There is a paucity of on-line biosensors to monitor the changes in the processes that occur in cultures of animal cells growing in a bioreactor. It would be preferable for such a probe to measure a metabolic variable. Since there is a sound theoretical basis for supposing that heat flux is a reflection of the overall metabolic flux, it was decided to explore the possibilities of marrying heat conduction calorimetry that measures heat flow rate with a dielectric spectrometer that estimates the viable biomass. The ratio of the two signals gives scalar heat flux. Previous work had shown that this variable could be used to validate the growth reaction of the cells in terms of the Mayer enthalpy balance method. It was revealed by the description of this reaction that the ratio of glucose to glutamine in the culture medium was incorrect. The finding stimulated the formulation of a revised medium that improved the specific growth rate of CHO320 cells producing recombinant interferon-γ (IFN-γ), as well as allowed a considerably greater yield of the target protein. In addition, the glucose and lactate fluxes decreased, indicating that there was less demand for biosynthetic precursors from glucose.

Keywords: Heat flux; Genetically engineered cells; Continuous culture; Fed-batch culture; Medium optimisation

1. Introduction

The production of medically important target proteins such as cytokines, monoclonal antibodies and vaccines from animal cells [1] has exposed a weakness in biochemical engineering because there is a lack of robust and reliable biosensors to monitor the processes in the bioreactor [2]. In addressing this problem, it was realised that there is an obvious advantage in monitoring a variable that indicates the state of cellular metabolism and it has been shown on theoretical grounds that the heat flow rate ($\Phi = dQ/dt$) measures the metabolic activity [3]. In order to make this measurement on-line, however, either the bioreactor must also be a calorimeter, a solution favoured in von Stockar's laboratory [4], or the cell suspension must be pumped to a flow calorimeter. The latter is the chosen method in this laboratory [5] and it was quickly realised that the heat flow rate obtained with a heat conduction microcalorimeter [6] was more sensitive to changing metabolic conditions in the culture than the growth curve constructed by cell counts. This meant that more information could be derived from the probe if the signal for the heat flow rate was made specific to the amount of biomass to give the scalar flux.

* Corresponding author. Tel.: +44-(0)1970-622333; fax: +44-(0)1970-622350.
E-mail address: rbk@aber.ac.uk (R.B. Kemp)

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It was already known that the capacitance (C) signal from a commercially available dielectric spectrometer [7] operating at a set radio frequency (0.5 MHz) was directly related to the quantity of live cells. Guan et al. [8] used flow cytometry on mammalian cells in suspension to confirm the precise relationship that the difference in the capacitance signal from that of the culture medium was due to the volume fraction of the viable cells. This estimation was chosen in preference to an assessment of the combined number of living and dead cells by laser nephalometry [2] because it was considered more relevant to the industrial question of cell productivity. The bioreactor software was programmed so that each of the two signals was smoothed on-line by the moving spot technique and then the ratio of heat flow rate to capacitance gave a continual trace for heat flux ($J_{\Phi/C}$).

The model system for studying the validity of the heat flux probe was CHO320 cells genetically engineered to produce constitutively the cytokine interferon-γ (IFN-γ) [9]. Changes in the concentrations of the major substrates and products, including IFN-γ, were compared with the continuous trace of heat flux [8]. It was concluded that there was indeed evidence for the hypothesis [3] of a monotonically increasing relationship between them, with heat flux being a function of the material fluxes. It was cautioned, however, that the only rigorous experimental proof of the relationship would be to set up continuous cultures at different dilution rates to obtain a series of steady state conditions for metabolism.

The analyses of materials and the heat flux measurement opened the twin possibility of expressing growth in terms of a chemical reaction and then validating the formulation of it by the enthalpy balance approach. It was Battley [10] who first advocated the description of growth by stoichiometric equations with biomass as a product. This is rather simple for microbes in that they usually grow on one carbon and one nitrogen source but animal cells have a variety of substrates for a nexus of interconnected catabolic and anabolic processes. It turns out, however, that the inputs for the growth reaction can be simplified to the two major carbon sources, glucose and glutamine, together with oxygen; and the outputs can to limited to the combined biomass and IFN-γ, with lactate, carbon dioxide and water [11]. The use of the Mayer enthalpy balance method [12] to validate the reaction has been used previously for the growth of yeast [13]. This organism has a relatively simple metabolism that is stable until the point of substrate depletion, unlike animal cells whose metabolic processes are very susceptible to change, depending on the relative availability of the substrates and on the environmental conditions. Thus, it is not possible to construct one reaction to describe growth but rather there must be a series of stoichiometric reactions over the entire growth period [14]. As an example, the reaction authenticated by the enthalpy balance method for the period between 51 and 76 h when there was strong growth was

$$\begin{aligned} C_6H_{12}O_6 + 0.377C_5H_{10}N_2O_3 + 0.44O_2 \\ = 3.52CH_{2.0}O_{0.82}N_{0.18} + 1.3C_3H_6O_3 \\ + 0.46CO_2 + 0.12NH_3 + 0.29H_2O \end{aligned} \quad (1)$$

The most revealing aspect of this reaction is that the cellular requirement for glucose and glutamine at this particular stage in growth is 2.65:1, whereas the medium contains 5.5:1. Although other time periods during growth show slightly different ratios [15], at no time do they actually approach the ratio in the medium. This information gave the impetus to improve the medium in several ways, not only by just altering the amount of glutamine [16]. A brief overview of the results is presented in this paper in order to demonstrate the efficacy of the improved medium in relation to steady state experimentation.

The most important contribution a metabolic probe can make to the industrial scale of animal cell culture is to control the fed-batch type in which small amounts of nutrient are added to the culture when there is retardation in growth [17]. In the simplest case, cell counts are taken at regular intervals, and when the increase in numbers slows down, more nutrients are added to the culture [18]. However, as stated above, reduced growth is a relatively late event in the metabolic decline of cells [8] and it would be preferable to use a more sensitive indicator, such as heat flux. For this variable to constitute an authentic probe, the theoretical proof from irreversible thermodynamics that heat flux is a function of the material fluxes must be backed by experimental evidence. This requires continuous cultures to be set up with a series of different dilutions to show that there is a set of metabolic steady states [19], such as has been done

in the past for CHO320 cells in glucose-limited culture [20]. Once the relationship has been shown, the stoichiometric nutritional requirements must be calculated for the cultures. Only then will it be justifiable to set up fed-batch cultures that have heat flux as the controlling variable.

The aims of this paper are to (i) prove that the heat flux variable is a function of substrate consumption fluxes; (ii) show by the heat flux probe that continuous cultures are at steady state; and (iii) calculate the stoichiometric nutritional requirements for these cultures and apply them to control nutrient feeding by heat flux.

2. Experimental

2.1. Cell culture

The suspension-adapted, genetically engineered CHO320 cells were grown as a 1.5 l tank culture in a controlled Applikon Bioreactor System (Applikon, Glous., UK) with the parameters of oxygen set as 55%, pH at 7.25 and temperature at 37°C with monitoring by suitable probes [8]. The revised culture medium was based on RPMI 1640 (Life Technologies, Paisley, UK) and contained many of the original components [9], but now they were in revised quantities (see Table 1 and [16,21]). For the continuous culture experiments, the medium was drawn by an Applikon pump from a reservoir to the bioreactor equipped with an overflow. It was also used to feed the nutrient cocktail (glucose, 50 mM; glutamine, 16 mM, in the improved medium) to the cells. The amplification of the co-transfected *dhfr* and human *IFN-γ* genes was made by selection with 0.1 μM methotrexate.

2.2. The on-line biosensors

The heat flux probe consisted of two distinct instruments. The Thermometric thermal activity monitor (TAM, Thermometric AB, S-17561 Järfälla, Sweden) was fitted with a custom-made flow module with all stainless steel tubing (1.5 mm ID), including the downward flow, coiled measuring vessel that has a physical volume of 1 cm^3 [22]. It was sterilised before use with 70% ethanol followed by a great excess of sterile water. The microcalorimeter acted as an on-line and ex situ device in that the cell suspension was circulated through the flow calorimeter by pumping it at 100 $cm^3\ h^{-1}$ with a Jubile peristaltic pump (H.J. Guldener, 8047 Zürich, Switzerland) equipped with Viton low diffusivity tubing. The system was calibrated electrically each day with a 50 Ω resister using the Joule effect and also periodically by a chemical reaction, the slow imidazole-catalysed hydrolysis of triacetin [23]. At the flow rate of 100 $cm^3\ h^{-1}$ used for these experiments, the thermal volume was 1.05 cm^3. This close approximation is not always the case and slower pumping rates can produce an overestimate of thermal volume [24].

Table 1
Comparison of the compositions of the improved and the original media for growing CHO320 cells in suspension

Medium components	Original	Improved
Glucose	10.5 mM	10.5 mM
BSA	5.0 g dm^{-3}	7.5 g dm^{-3}
Insulin	5 mg dm^{-3}	5 mg dm^{-3}
Human transferrin	5 mg dm^{-3}	5 mg dm^{-3}
Gentamycin	50 mg dm^{-3}	50 mg dm^{-3}
Putresine	1 μM	1 μM
$FeSO_4$	10 nM	10 nM
Na_2SeO_4	10 nM	10 nM
$CuSO_4$	10 nM	10 nM
$ZnSO_4$	3.0 μM	3.0 μM
Methotrexate	0.1 μM	0.1 μM
Pentex ExCyte	None	5 (v/v)%
Sodium pyruvate	1 mM	2.5 mM
Glutamine	2 mM	3.3 mM
Alanine	0.1 mM	0.2 mM
Asparagine	0.38 mM	0.5 mM
Arginine	1.15 mM	1.15 mM
Aspartate	0.15 mM	0.5 mM
Cystine	0.21 mM	0.5 mM
Glutamate	0.14 mM	0.5 mM
Histidine	0.1 mM	0.5 mM
4-Hydroxy Proline	0.15 mM	0.5 mM
Proline	0.17 mM	0.5 mM
Glycine	0.13 mM	0.5 mM
Isoleucine	0.38 mM	0.5 mM
Leucine	0.38 mM	0.5 mM
Lysine	0.22 mM	0.5 mM
Methionine	0.10 mM	0.5 mM
Phenyl alanine	0.09 mM	0.5 mM
Proline	0.17 mM	0.5 mM
Serine	0.29 mM	0.5 mM
Threonine	0.17 mM	0.5 mM
Tryptophan	0.024 mM	0.5 mM
Tyrosine	0.11 mM	0.5 mM
Valine	0.17 mM	0.5 mM

The second component to the heat flux biosensor was the Aber Instruments Viable Cell Monitor (Aber Instruments, Aberystwyth, UK) operating at 0.5 MHz in the β-dispersion [7]. The autoclavable probe of the on-line, in situ instrument was placed in the bioreactor. The two signals from the probe are capacitance to measure biomass and conductivity that must remain constant throughout the experiment because of cross-talk with the capacitance signal. While the analogue signal from the TAM was A/D converted by the one spare channel in the Applikon Biocontroller, a separate Applikon multi-channel AD/DA converter PCL-812PG PC-Labcard converted the two from the VCM. All three digital signals were processed in the Applikon BioXpert software and those for heat flow rate and capacitance were smoothed by the moving average technique before division obtained the ratio. As there was still some noise in the combined signal, for the feeding strategy, the software was programmed only to instruct the pump to give a pulse of the medium if there were an average decrease of 5% over a 1 h period.

2.3. Off-line measurements

The cell number concentration was estimated using a Coulter Counter, Model D (Coulter, Luton, UK) and the percentage viability was assessed by staining with fluorescein diacetate (green for live cells) and ethidium bromide (red for dead cells) [25]. Oxygen uptake rate (OUR) was measured using a high resolution Oroboros Oxygraph twin channel respirometer with DatLab software (Oroboros, Innsbruck, Austria) [26]. The cell suspension, 2.3 cm^3, was added to each chamber and stirred with a magnet at 400 rpm.

For the materials, the ammonium concentration was measured by a Radiometer PHM95 pH/ion meter (Radiometer Analytical A/S, DK-2400 Copenhagen, Denmark) using an ammonium selective electrode, the IFN-γ concentration was assayed by a specific antibody ELISA technique [9] and the concentrations of glucose, glutamine and lactate were estimated by conventional HPLC with Refractive Index (Cecil Instruments, Cambridge, UK) and Ultra Violet (LKB Produkter AB, Bromma, Sweden) detectors.

3. Results and discussion

The revised medium (see Table 1) was an improvement in terms of cellular growth and production of the target protein, as described in detail by Guan and Kemp [16]. The fluxes for cells cultured in the revised medium (Fig. 1) were compared with those of cultures

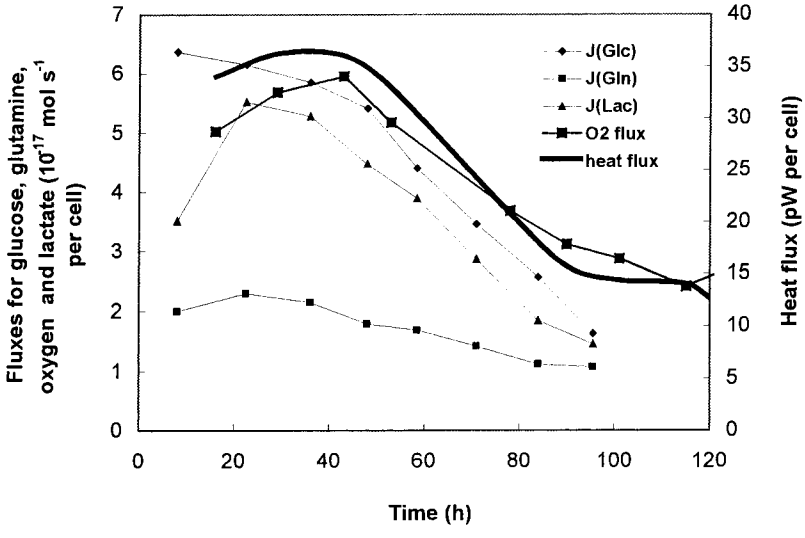

Fig. 1. Fluxes for the utilisation of glucose, glutamine and O_2 and the production of lactate in a batch culture of CHO320 cells over a period of 120 h, with the data obtained by off-line analyses at discrete time intervals. The data should be compared with those of heat flux obtained on-line.

in the old one. In brief, the changes in the ingredients were responsible for a 75% increase in specific growth rate and a 25% improvement in IFN-γ flux while causing decreases in glucose (30%) and lactate (50%) fluxes. It can be supposed that the reason for these changes was, first, that the increased glutamine content of the revised medium allowed the cells to grow for a longer period of time. Compared to the material fluxes in the original medium, the glutamine flux was only slightly reduced, indicating that, even in the original medium, most of it was utilised in biosynthesis. Secondly, the greater amounts of the other amino acids permitted anabolic processes without resort to biosynthetic precursors. The evidence that this was the case stemmed from the reduced glucose and lactate fluxes. The fact that the latter decreased more than the former might suggest that there was less glutaminolysis to provide the other amino acids and energy, though radioisotopic tracing would be needed to prove this supposition. In any case, the lower level of lactate in the culture medium would reduce the possibility that the toxic effects of this catabolite [27] inhibited the cell growth.

It is likely that the increased amounts of co-factors and hormones helped the improvement in cell growth, but the most encouraging cellular response was in the considerably greater production of IFN-γ (see Fig. 2) because the target protein is the sole reason for the industrial process. Other attempts at improving the medium for cell culture, either the statistical approach adopted by Castro et al. [28] for CHO320 cells or the exhaustive stoichiometric analysis undertaken for hybridoma cells by Xie and Wang [29], have proved similarly useful exercises. The current study combines these two methods in an empirical approach [16] and has resulted in the greatest improvement in yield but there has not yet been the opportunity to discover if the quantitative increase has been complemented by betterment in quality. It is generally considered that the superior environmental circumstance [30], as well as the addition to the medium of lipid supplements such as Pentex ExCyte [31,32], reduce macro- and microheterogeneity. The improved medium had been formulated with these prospects in mind.

The empirical evidence of a monotonically increasing relationship between heat flux and the material fluxes [8] stems from knowledge of the advancement of a reaction, ξ. As demonstrated previously by Guan and Kemp [16], this is defined as

$$\xi = \frac{n_B}{v_B} \tag{2}$$

where v is the stoichiometric coefficient, n is the change in molar number, and B is the amount concentration of the entities (reactants and products). The metabolic (flow) rate is more generally known as the

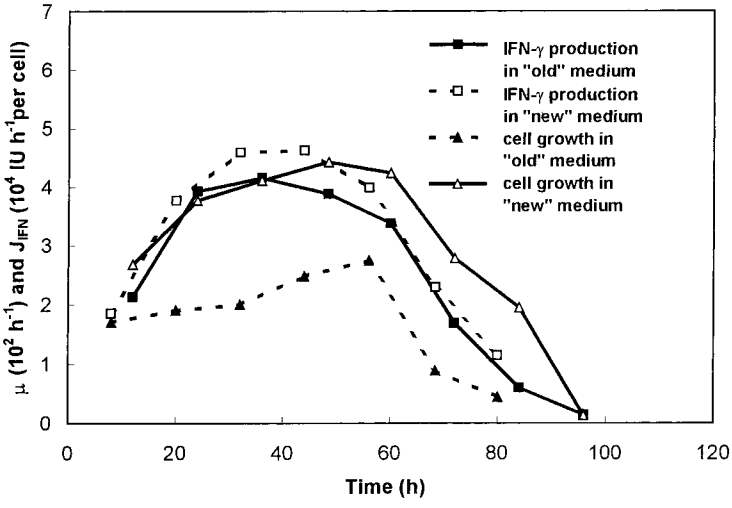

Fig. 2. The effects of an improvement of medium on both specific cell growth rate and interferon-γ production flux during batch culture of CHO320 cells.

rate of conversion, $d\xi/dt$, a kinetic term that can be made size-specific as the metabolic flux (J_B):

$$J_B = \frac{d\xi}{X\,dt} \tag{3}$$

where X is the amount of biomass. From the monotonic relationship between the metabolic flux and the stoichiometry of the growth reaction, it follows that

$$\frac{1}{X}\frac{d\xi}{dt} \leftrightarrow \vec{v} \tag{4}$$

Since heat flux, J_{th} is a form of metabolic flux [16], Eq. (4) can be converted as

$$v_i = f(J_{th}) \tag{5}$$

The strength of this relationship is illustrated from data for the utilisation of glucose and glutamine and for the production of lactate, ammonia and the cellmass in relation to the heat flux for the following stoichiometric ratios in the period 0−100 h shown in Fig. 1:

$$v_{Glc} = 0.0581J_{th}^2 - 2.095J_{th} + 19.7 \tag{6}$$

$$v_{Gln} = 0.0279J_{th}^2 - 1.05J_{th} + 9.86 \tag{7}$$

$$v_{Lac} = 0.015J_{th}^2 - 0.334J_{th} + 2.60 \tag{8}$$

$$v_{NH_3} = 0.0245J_{th} - 0.223 \tag{9}$$

$$v_{cell} = 0.180J_{th}^2 - 6.39J_{th} + 56.9 \tag{10}$$

From Eqs. (6)–(10), it can be seen (Fig. 3) that, for the specified range, heat flux is a monotonically increasing function of these ratios, thus further substantiating its validity as a metabolic probe. This means that it can be used in fed-batch cultures to indicate the point at which cells should be fed nutrients. As stated earlier, at present, off-line samples are taken for chemical analyses and/or cell counts and changes in the appropriate flux and/or cell number are used as an indicator of the need for nutrients [18,29,33].

Before demonstrating the value of heat flux as the controlling variable in feeding nutrients to cells, it was necessary to set up continuous cultures to (i) show at the different dilution rates that the various metabolic fluxes are at steady state; and (ii) obtain the stoichiometric nutritional requirements at each metabolic rate. The heat flux probe gave a series of plateaux at the different dilution rates and this confirmed that the

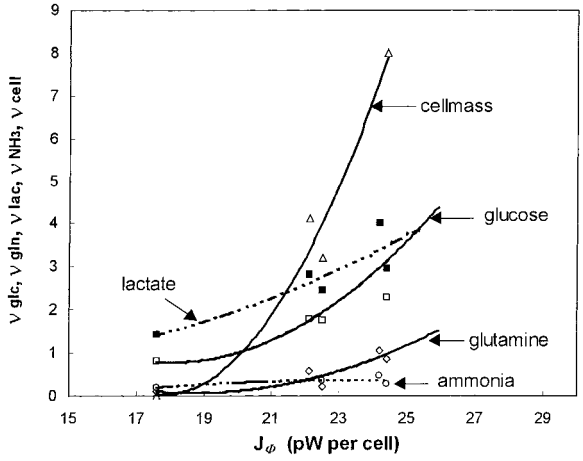

Fig. 3. The heat flux over a specified set of values is compared against the stoichiometric coefficients for glucose, glutamine, lactate, ammonia and viable cellmass. The stoichiometric coefficient for oxygen was set as unity. The data for these cases (see Eqs. (6)–(10)) show the relationship of heat flux to glucose (□), glutamine (▲) consumption fluxes, and to lactate (■), ammonia (○) and viable cell (△) production fluxes.

metabolic flux was at steady state for each of them. As illustrated in Fig. 4, the value for the metabolic flux increased with increasing dilution rate. It was also observed that, for a given steady state, glucose was at a low concentration and glutamine was depleted entirely in most cases. The concentration of lactate appeared to vary for the dilution rate, D=0.016 h^{-1}. However, the constant heat flux for each of the selected periods (they lasted from 4 to 7 days) means that the steady states were properly achieved without long transition periods. In summary, the data depicted in Fig. 4 give the required proof of the strict relationship between the material stoichiometric coefficients and their equivalent in terms of heat.

With this reassurance, the fed-batch experiments were conducted with the biosensor signal in the Applikon software to trigger the feeding of the nutrient cocktail to the cells. The results represented in Fig. 5 showed that feeding effectively restored the level of metabolic activity. However, the fact that the response had to be dampened to prevent nutrient addition occurring due to electronic noise meant that the cells received fresh medium slightly later than would be ideal. This is reflected in the small downward trend in the heat flux over the many hours of the

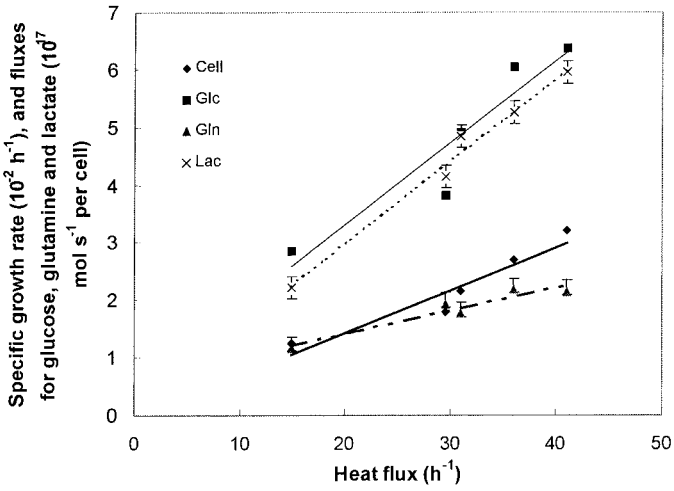

Fig. 4. The cell specific growth rate, and fluxes for glucose, glutamine and lactate were correlated to heat flux at different dilution rates in a continuous culture.

experiment and contrasts with the rapid decline in heat flux for batch cultures after 60–80 h in batch culture. The effect became more marked at cell concentrations below approximately 10^6 cm^{-3} and it was then not possible to arrest what was found to be the terminal decline of the culture (not shown). It is suggested from the present results that, once a culture system has been defined in metabolic terms, the heat flux probe is an ideal way for early intervention to control feeding by an automated on-line technique. The probe is universally applicable to all animal cell cultures because every metabolic state can be represented by a set of stoichiometric coefficients that includes the heat equivalent of such a coefficient.

In conclusion, the work described in this paper has demonstrated that cellular heat flux measures the overall metabolic 'activity' or, more correctly, the metabolic flux. This qualifies the heat flux biosensor as a powerful tool to (1) monitor the metabolic status of cells in batch culture; (2) aid in the design of the

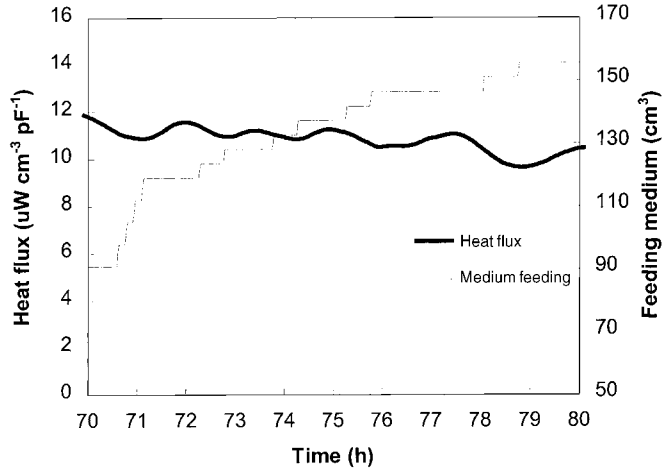

Fig. 5. This shows a small section of the heat profile for a fed-batch culture (from 70 to 80 h) to illustrate that the medium feeding was triggered by the declining heat flux values over a 1 h assessment period. The heat flux was restored, to a varied extent, by this feeding strategy.

culture medium; (3) monitor the attainment of steady state in continuous culture; and (4) act as a control variable in fed-batch culture. It is indeed a useful tool.

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