BIOPROCESS ENGINEERING – BIOPROCESS ANALYSIS THROUGH CALORIMETRY AND BIOTHERMODYNAMICS

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Contents

Summary:

Gibbs energy dissipation often in the form of heat energy is an explicit consequence of the second law of thermodynamics and a general feature of bioprocesses. This is because organisms converting CO_2 or organic matter into biomass or any bioproducts cannot use all the Gibbs energy provided by the assimilated substrates or other energy sources, i.e. essential fractions of the Gibbs energy often are wasted as heat. This heat production reflects metabolic shifts in real time and can be measured non-invasively by direct calorimetry. By applying the first law of thermodynamics, this technique permits the user to test if process heat is in accordance with assumed metabolic pathways and, if not, thereby to detect unknown reactions. Heat production is incomparably the ideal

indicator for monitoring and analyzing bioprocesses. Hitherto, technical obstacles have prevented the broad distribution among the scientific community of this powerful methodology. However, recent developments removed from calorimetry the old weaknesses and qualify the technique for:

- high-throughput measurements,
- analyzing bioprocesses in bioreactors of different sizes (µL to m^3),
- monitoring of trace pollutant biodegradation and
- bioprocess control even under "dirty" technical conditions.

1. Introduction

The phenomenon of life itself is intimately and inherently tied to Gibbs energy dissipation often in the form of heat energy (J). The amount of it depends on the growth and product formation stoichiometry and its production rate (J s^{-1}, W) additionally on the kinetics. The measurements of this rate, i.e. calorimetry, combined with further bioprocess analysis, provide insights to the on-going bioprocess (Bioprocess Analysis).

Calorimetry is suited for Bioprocess Control even for such complex systems that have growing species, food chains, different phase transitions, and/or large interference with physical processes, e.g. aeration, stirring, and/or mixing, which are difficult to interpret by thermodynamics. The advantages of thermal sensors for the control of simple bioprocesses control are fast response times, robustness and increasing sensitivity with any up scaling due to increasing the ratio of heat producing volume to heat exchanging surface.

The heat production rate – requiring sophisticated measurement techniques at the bench scale – can develop into a real problem at large scale because the heat exchange with the environment is negligible (i.e. adiabatic mode) at this scale. This means that all the heat produced by the culture will contribute to an increase of temperature and a deactivation of the microbial catalyst, unless the bioreactor contains appropriate cooling facilities. The quantitative knowledge of the microbial heat production rate is therefore crucial to successful Bioreactor Design [see also – *Bioreactor Types*].

The degradation of high value chemical energy from nutrients as substrates (low entropy) into heat or disordered structures (high entropy) is a prerequisite for life. Perception of the relationship between Gibbs energy dissipation and heat production helps in the general understanding of the Driving Forces of Bioprocesses. The more Gibbs energy of the nutrients is dissipated, the less energy will be retained for the formation of biomass or products and thus the lower will be the respective yields. By following the principles of irreversible thermodynamics, it can be seen that the rates of bioprocesses are influenced by the amount of the driving force, i.e. the Gibbs energy dissipation. In evolutionary terms, Nature has had to find a compromise between high yield and high rates. The important practical consequences of a better understanding of the thermodynamics of microbial growth and product formation are predictions about maximum possible yields, maximum practical yields, maintenance coefficients, threshold concentrations and even maximum growth rates.

The need to quantify the relatively small biological heat production in the microwatt scale spurred the development and perfection of the modern isothermal microcalorimeter (ITMC). Due to its extraordinary sensitivity, it has contributed a great deal to biochemical and biophysical research. Nevertheless, microcalorimetric research has hardly touched progress in bioengineering progress in recent years. The reasons are:

- difficulties in agitation and aeration;
- complexities in the integration of additional microsensors like pH, Redox, UV/VIS or pO_2; and
- small sample volumes due to the relatively small calorimetric vessel.

These experimental limitations in microcalorimetry lead to experimental conditions which often diverge in essence from the real process conditions. However, the main weakness of the conventional ITMC, as well as of the bench scale calorimeter, is the restriction in data collection because it is possible only to follow relatively few bioprocesses in parallel whereas, for instance, modern biotechnological strain optimizations are nowadays performed in up to 1536 well plates. Furthermore conventional calorimeters are predominantly designed and produced for only a few applications resulting in relatively high prices for good calorimeters. Therefore, calorimetry will never tap its full potential until at least a few of these weaknesses are overcome.

However, the recent trends in calorimetric developments such as

- megacalorimetry,
- miniaturized calorimetry (chip-, integrated circuit-, nano-calorimetry),
- high-throughput calorimetry (enthalpy arrays),
- ultra-sensitive calorimetry and
- photocalorimetry

promise to remedy the deficiencies and provide calorimetry with a brilliant future as a scientific tool as well as a technical sensor. The roots, the state of the art and the potential of calorimetry will be discussed in the following sections.

2. Roots of Modern Biocalorimetry

For centuries or even millennia, fire has been seen as a symbol of life and its extinction a synonym for death. This may explain why living systems have been the objects for calorimetric experiments and thermodynamic interpretations since the early days of this technique. Even the emerging trends of recent calorimetry to analyze and control bioprocesses stand in the tradition of a long history of thermal analyses.

Ever since the first calorimetric experiments with guinea pigs more than 200 years ago by Lavoisier (1780) and Crawford (1777), it has been recognized that the heat produced from living matter is a real time measure of metabolic activity. A more quantitative view based on studies of horse physiology arose when Robert Mayer (1842) established the law of conservation of energy (The First Law of Thermodynamics). Germain Henri Hess' discovery of the additivity of independent determined enthalpy is a special statement of the First Law allowing the quantitative interpretation of biocalorimetric

experiments. Max Rubner demonstrated (1890), using a dog's metabolism, that Hess' law is not only applicable to chemistry, but also to biology. The conclusion that there were no principle thermodynamic differences between inanimate and living systems paved the way for the further development of biocalorimetry, e.g. as a tool for online stoichiometry or for metabolic flux analysis. Thus *quantitative calorimetry* is an important recent trend. It combines calorimetry with other measurements either on/at-line (Fourier transform infrared spectroscopy, dielectric spectroscopy, optical density, flow injection analysis, gas analysis) or off-line (HPLC, enzymatic analysis of metabolites and products, flowcytometric analysis of cell states, etc) in order to establish that the growth stoichiometry is complete and, if not, to calculate the missing part using enthalpy and elemental balances.

The first successful calorimetric experiments used macroscopic live forms, but it is not the heat production rate per unit living mass per se that makes calorimetry with the biotechnologically more important microscopic life more difficult. In practice, exponentially growing yeast (250 W kg^{-1}) and even resting yeast cultures (5 W kg^{-1}) show much higher specific heat production rates than Guinea pigs (3 W kg^{-1}) or humans (1.3 W kg^{-1}). In cultures, the difficulty results from the high heat storage capacity of the media which usually contain low volume fractions of microorganisms. The heat capacity of air (1.2 kJ m^{-3} K^{-1}) is about three orders of magnitude lower than that of water (4200 kJ m^{-3} K^{-1}) or soils (580 – 3100 kJ m^{-3} K^{-1}) (T=298 K, P= 101 325 Pa). As early as 1856, Pierre Dubrunfaut found an impressively simple solution to the problem and he is now reputed as the father of what these days is the quite topical *Megacalorimetry*, i.e. calorimetry in reaction tanks of several hundred litres or even m^3 used for process control. Dubrunfaut performed an alcoholic fermentation of 2559 kg sugar in a huge oak vessel of 21 400 L volume. In this way he maximized the ratio of heat-producing volume to heat-exchanging surface and observed a temperature increase from 23.7 ^oC to 33.7 ^oC during 4 days of fermentation at ambient temperatures between 12 ^oC and 16 ^oC. Taking into account heat loss by radiation, convection and evaporation and heat storage by the wood, he calculated a fermentation heat of -94.9 kJ mol^{-1} glucose equivalent, a number surprisingly close to the now established value of -138.6 kJ mol^{-1}. New, more sensitive temperature probes, less temperature dependent electronic devices and progress in real time data acquisition further the cause of *Megacalorimetry* as the second recent trend in calorimetry.

Whereas up scaling reduces the influence of environmental fluctuations, reduction in calorimeter size diminishes the amount of heat energy needed for the calorimetric device while speeding up the measurement. Developments towards short response and equilibration times, e.g. *Miniaturized Calorimetry* (resulting in integrated circuit (IC) calorimetry) are a third recent trend in calorimetry development. The principle of IC-calorimetry is illustrated in figure 1. The first thin-film calorimeter was developed during the early seventies but recent, new techniques of printed circuit design have boosted the development of them, as testified by the publication of more than 70 papers in the last decade. As IC calorimeters nowadays measure a few nW they are also nicknamed *Nanocalorimeters*. The expected progress in IC calorimeter development will lead to: (i) extremely small thermal detectors; (ii) minute sample mass requirements; (iii) increased sensitivity; and (iv) much shorter response and

equilibration times. In particular, immediately responding small detectors are crucial for the integration of calorimetric sensors into established bioreactors.

At present, even nanocalorimetry is too slow to compete with the robotic monitoring of metabolic activities of cells growing in 384- or 1536-well microtitre plates, used as a diagnostic tool in the pharmaceutical industry or in biotechnology. Calorimetry suffers from low throughput in comparison to such rivals. Therefore, a fourth recent trend in biocalorimetry is the development of the *High-Throughput Calorimeter*, i.e. the so-called enthalpy arrays.

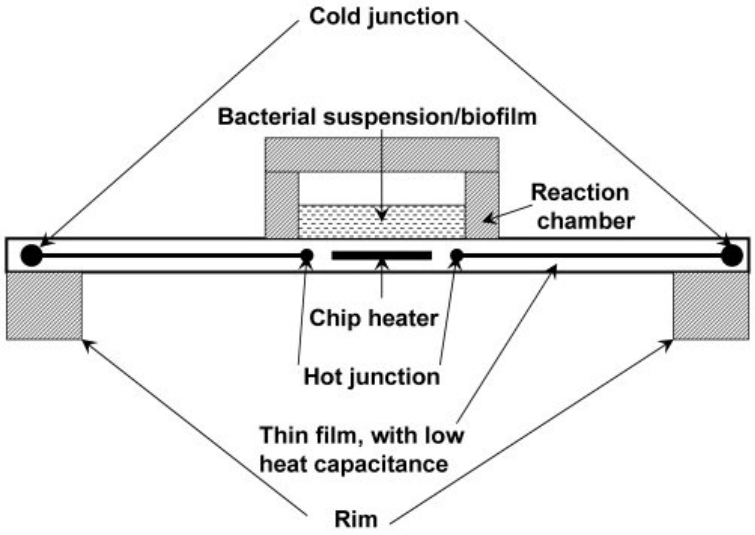

Figure 1: Measurement principle of a chip calorimeter.

On another front, there is real concern about the potentially deleterious effect on human health of micropollutants or of hydrophobic organic compounds (HOC) due to their ubiquity in the environment and their persistence. HOC's dissolving only slightly in aqueous phases tend to interact with non-aqueous phases and, as a consequence, are poorly bioavailable for microbial degradation. To measure this, the novel *Ultra-Sensitive Calorimetry* has to be developed in order to gain real time information on the kinetics and the physiology of bioconversion of HOC traces in homogenous aqueous as well as in heterogeneous phases.

The overwhelming majority of calorimetric measurements have been of metabolic heats originating from chemical bonding energies and have disregarded other energy forms. However the life on earth in its present form is unthinkable without the energetic utilization of light. Magee and co-workers recognised this absence as early as 1939 and developed small-scale *Photocalorimetry*. On another topic, electrical energy drawn from microbial fuel cells (MIC) or put to anaerobically growing cells to change the redox balance have been known and expected to influence energy balances since the early 1900s. However, no calorimetric measurements considering this energy form are known to the authors despite its importance and long history. Therefore, calorimetric techniques are urgently required that take energy forms other than chemical energy into account.

In the following, the status quo and the potential of the six recent trends in biocalorimetry will be discussed in more detail.

3. Information Content of Calorimetric Signals

3.1. Stoichiometry and Kinetics of Microbial Growth and Product Formation

The most important information for an engineer designing a biotechnological process is its stoichiometry and kinetics. Both characteristics are tightly connected with the heat production rate. However, to analyze this correlation, the dynamic energy balance for the open technical or natural system of interest has to known. Eq. (1) describes a dynamic energy balance for such a system:

$$V \rho \bar{c}_p \frac{dT}{dt} = P + \dot{W} + \sum_e \sum_i \dot{n}_{i,e} \bar{c}_{pi,e} (T_e - T) - \sum_j \Delta_R H_j \dot{\xi}_j \qquad (1)$$

The term $V \rho \bar{c}_p \frac{dT}{dt}$ describes the heat accumulation in the whole system as the increase of temperature *T* versus time *t*. $\bar{c}_P$ is the mean heat capacity of the system, *V* its volume and ρ its density. Heat *P*, work $\dot{W}$ and material fluxes $\dot{n}_e$ are exchanged with the environment. The exchanged material of the species *i* transports energy proportional to the heat capacity at constant pressure $\bar{c}_{Pi,e}$ and to the temperature difference *(T_e – T)* of the respective entry or exit port *e*. The reaction term $\sum_j \Delta_R H_j \dot{\xi}_j$ correlates the energy rate generated or consumed by the reaction *j* with the respective reaction enthalpy $\Delta_R H_j$ and the absolute rate of the reaction $\dot{\xi}_j$. The reaction term includes metabolic and chemical reactions as well as phase transitions.

It is of practical importance that all the components in Eq. (1) can be measured and balanced in order to find out how complete or correct are the assumptions about the technical or natural system. The first modern application of this principle in biotechnology, 150 years after Dubrunfaut's pioneering work around 1856, became known as balancing calorimetry. Recently, several research groups have improved bench scale and industrial scale calorimetry. Their technical developments were aimed at reducing the complexity of Eq. (1) by eliminating abiotic sources of error. This was achieved by: (i) reducing the heat accumulation term $V \rho \bar{c}_p \frac{dT}{dt}$ through operation under isothermal conditions; (ii) reducing the convection term $\sum_e \sum_i \dot{n}_{ie} \bar{c}_{pi,e} (T_e - T)$ by thermostating the entering and leaving streams; and/or (iii) keeping $\dot{W}$ (predominately stirrer work) constant. The facilities of any "off the peg" bioreactor can be employed for heat flux measurements by using the above discussed achievements combined with modern temperature probes to give less overall expenditure in terms of both labor and finance. Such an improvised calorimeter works with a sensitivity of approx. 50 mW L^{-1}.

Figure 2 comparing the calculated with the measured heat production rate shows the applicability of such a non-conventional calorimeter.

Under ideal conditions, the produced heat reflects nothing but the progress of all ongoing metabolic or chemical reactions (Eq. 2):

$$P \approx \sum_j \Delta_R H_j \dot{\xi}_j \qquad \text{with} \quad \dot{\xi}_j = r_{ij}\, \nu_{ij} \tag{2}$$

where r_{ij} and ν_{ij} are the rate and the stoichiometric coefficient, respectively, of the component *i* participating in reaction *j*.

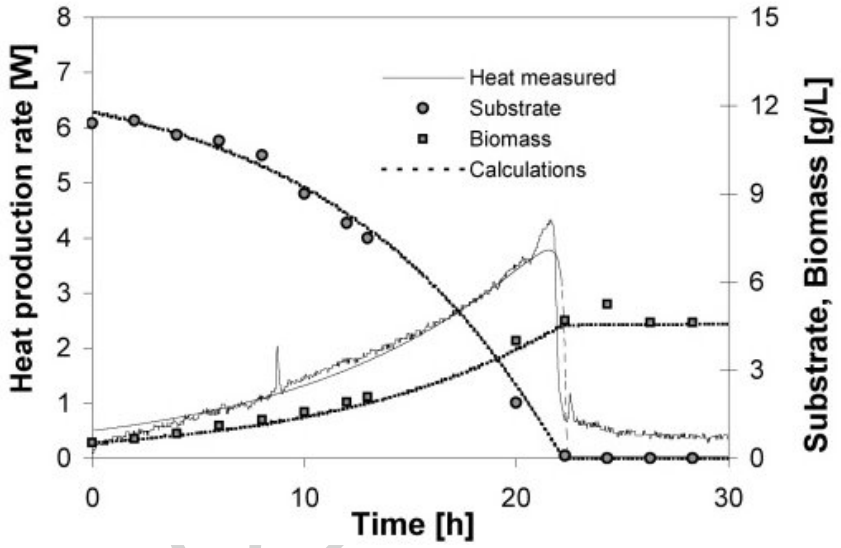

Figure 2: Test of the bioreactor used as an "off the peg" calorimeter. Comparison of the calculated with the measured heat production rate for the aerobic growth of *H. elongata* on glycerol.

The heat of non-metabolic reactions, e.g. neutralization heats, can be obtained by the titrimetric consumption of alkali or acid using tabulated values for correction. The metabolic heat production can then be correlated with the stoichiometry of microbial growth and product formation processes. The microbial growth reaction in its simplest case can be described stoichiometrically by Eq. (3):

$$Y_{S/X}\, C_{S1}H_{S2}O_{S3} + Y_{N/X}\, NH_4^+ + Y_{O/X}\, O_2 \rightarrow$$

$$C_{X1}H_{X2}O_{X3}N_{X4} + Y_{H2O/X}\, H_2O + Y_{CO2/X}\, HCO_3^- + Y_{H/X}\, H^+ \tag{3}$$

where $C_{S1}H_{S2}O_{S3}$ is the applied carbon source, NH_4^+ and O_2 stand for the nitrogen source and the terminal electron acceptor, respectively, and $C_{X1}H_{X2}O_{X3}N_{X4}$ is the elemental composition of the biomass. The elemental composition of bacteria has been averaged to $C_4H_8O_2N_1$ or more recently to $CH_{1.84}O_{0.53}N_{0.23}$. Both values can be applied for stoichiometric considerations very accurately if no exact elemental composition is available. The six unknown yield coefficients ($Y_{S/X}$, $Y_{N/X}$, $Y_{O/X}$, $Y_{H2O/X}$, $Y_{CO2/X}$, $Y_{H/X}$) in

Eq. (3) are not independent from each other because they have to satisfy five balances (four elemental balances and one charge balance). This means that adding the enthalpy balance and measuring the reaction enthalpy of the growth process $\Delta_R H_x$ allows the calculation of each of the unknown yield coefficients. Two reference states for the enthalpies are commonly used for this purpose, namely the constituent elements of all the involved species (enthalpies of formation) and the completely combusted state (enthalpies of combustion, $\Delta_C H$). Using the latter simplifies the enthalpy balance (Eq. 4):

$$\Delta_R H_x = -(\Delta_C H_X + Y_{H/X}\,\Delta_C H_H - Y_{S/X}\,\Delta_C H_S - Y_{N/X}\,\Delta_C H_N) \qquad (4)$$

The combustion enthalpies of each species are tabulated in the literature (, e.g. see Domalski in the bibliography). There are also some combustion enthalpies of biomass of different organisms growing under different conditions available from the same source. If there are no data available, the combustion enthalpy can be estimated very accurately from the elemental composition applying the Thornton's rule or more recent correlations. The heat production rate P of a microbial growth process depends on the reaction heat of the growth process $\Delta_R H_x$ and on the biomass formation rate r_X (Eq. 5),

$$P = r_X\,\Delta_R H_x \qquad (5)$$

In the case of chemostatic growth Eq. (5) reads:

$$P = \frac{D\left(S^0 - S\right)}{Y_{S/X}}\,\Delta_R H_X \qquad (6)$$

The dilution rate D and the substrate concentration of the reactor input S^0 are predefined by the operator. The residual substrate concentration S is usually low in comparison to S^0 in chemostatic bacterial or yeast cultures and can thus be neglected in enthalpic or stoichiometric balances. Therefore, the measured P reflects $\Delta_R H_x$ and, using the balances of the enthalpy and the elements, provides the real time stoichiometry.

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Biographical Sketches

Thomas Maskow, born in 1962, studied Theoretical and Physical Chemistry at the Technical University "Carl Schorlemmer" Leuna-Merseburg (1983-1988). He achieved his Doctoral degree at the Martin-Luther University Halle-Wittenberg for the development of new analytical tools and thermodynamic models to characterize the phase behavior of crude oil or oil fractions under guidance of Prof. H. Kehlen and Prof. M. Rätzsch. Afterwards he directed the research division of a medium sized company (R. Meyer Laboranalysen und Umwelttechnik GmbH Zeitz) dealing with analytical services, costumer-specific developments and functioning as consulting engineer in the environmental field (1992-1996). 1996 he was offered the chance to establish and develop biocalorimetry and biothermodynamics at the UFZ Helmholtz Centre for Environmental Research. His work was strongly supported by the executives of the Department of Environmental Microbiology (Prof. W. Babel/Prof. H. Harms). In addition to active research in the fields of bioprocess control, extremophiles and stress biology he teaches biotechnology and biophysics at the nearby universities (Leipzig and Dresden). 2002 and 2004 he was invited to work as guest scientist at the Institute of Biological Sciences, University of Wales, Aberystwyth, UK (laboratory of the coauthor) and at the Institute of Chemical Engineering, Swiss Federal Institute of Technology Lausanne (EPFL), Switzerland (Laboratory of Prof. U. von Stockar). 2006 he qualified for university lecturer (Habilitation). He takes active part in different boards such as "Bioprocess Technology", "Biothermodynamics" and "Forum Biotechnology" at the DECHEMA (Society for Chemical Engineering and Biotechnology) and in the International Society of Biocalorimetry (ISBC). Since 2006 he is a member of the advisory board of the journal "Engineering in Life Sciences".

Richard Kemp, born in 1941, graduated with a B.Sc. (Hon) in Zoology from London University (Kings College) in 1963 before undertaking postgraduate studies in Cell Biology under Prof. Bryn Jones at the University of Wales, Aberystwyth, obtaining a PhD in 1966. After periods of postdoctoral research at the University of Uppsala, Biochemistry Department with Professor J. Hjertén (1966) on the electrophoretic separation of proteins and the University of Cambridge, Department of Radiotherapeutics with Dr G.V.F. Seaman (1967) on cell electrophoresis, he returned to Abersytwyth as the Scientific Research Council Assistant Director of Research in Cell Biology in 1967, principally working on cell-cell adhesion. He has remained there ever since firstly as a Lecturer (1971), then as a Senior Lecturer (1983) and finally as the Reader in Cell Physiology and Head of the Cell Biology Laboratory (1986). He has had periods of sabbatical leave at the Department of Biophysics, University of Kyoto, Japan with Professor T.S. Okada on cell adhesion (1972), the John Curtin School of Medicine, Canberra, Australia with Dr W. Field-Smith on the energetics of the adhesion process (1983), the Department of Zoology, University of Innsbruck, Austria with Professor Erich Gnaiger on the calorimetric measurement of metabolism in mammalian cells (1991) and the Department of Biochemistry, Russian National Academy of Sciences, Kazan, Russsia with Professor Lev Gordon on the photocalorimetric measurement of photosynthesis in microalgae (2000). Over the years, his scientific research has shifted from cell adhesion to cellular energetics. It is for his work on the latter that he was elected Fellow of the UK Institute of Biology, 1989, was awarded the Lavoisier Medal of the International Society for Biological Calorimetry, 1992, and gave the NATAS Award lecture at the 11th International Congress of the International Society for Thermal Analysis and Calorimetry, Philadelphia, USA, 1996. Thermochimica Acta Volume 394 as a festschrift was dedicated to him on the occasion of his 60th birthday, 340pp., and published exactly one year later (19 October 2002). Currently, he is working on the calorimetric measurement of metabolism in the bioprocesses of cells ranging from prokaryotic microorganisms to animal cells, but principally using photocalorimetry to study the photosynthetic process in microalgae at both the analytical and biotechnological scales.