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Thermochimica Acta 382 (2002) 229–237

thermochimica acta

www.elsevier.com/locate/tca

Calorimetric analysis of microorganisms in transient growth states to quantify changes of metabolic fluxes in response to nutrient deficiencies and osmostress

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Received 31 May 2001; received in revised form 19 July 2001; accepted 30 July 2001

Abstract

The catalytic potential of microorganisms is often limited by sub- or supra-optimal environmental factors, which frequently fluctuate with varying amplitude and frequency. In chemoorganoheterotrophic growth, microorganisms have to adjust their utilisation of Gibbs energy, and thus their heat flux and network of reaction fluxes, to cope with such fluctuations. A transient-state technique was developed to quantify changes of metabolism and to compare the enthalpy balance with the heat flux. This type of comparison can be used to assess the completeness of flux analysis. A special feeding system was designed to identify responses to specific environmental shortcomings. For this purpose, microorganisms were grown continuously in a bench-scale calorimeter and the concentration of a single component in the feeding stream was increased gradually, while all the other growing conditions were kept constant. The mathematical tools describing the concentration gradient and the microbial responses (i.e. key changes in metabolic rates) are presented. These equations were derived by solving the differential balances describing the concentrations of the substances varied in the feeding system, or similarly considering elements, substances and enthalpy in the calorimeter. The proposed method was tested on *Variovorax paradoxus* DMSZ 4065 and *Halomonas elongata* DMSZ 2581^T subjected to nitrogen shortages and halo-stress, respectively. The major responses considered were the synthesis of poly-3-hydroxybutyrate (PHB) in the first case, and the synthesis of a compatible solute, 1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid (ectoine), in the latter. The measured rates and fluxes were consistent with the enthalpy and elemental balances as well as theoretical expectations, indicating that this transient-state technique is valid for quantifying changes of metabolic fluxes in response to specific stresses.

Keywords: Calorimetry; Transient-state; Ectoine; Poly-3-hydroxybutyrate; Stress

1. Introduction

Chemoorganoheterotrophic bacteria have the potential to catalyse the degradation of harmful organics and synthesis of harmless, valuable products. However, this potential is seldom optimised in practical systems. The velocity and efficiency of conversion are usually limited by external physical factors. In some cases, the efficiency of the process is also restricted by adverse properties of the substrates and products, which may—depending on their concentration—inactivate and poison the catalysts. Thus,

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0040-6031/02/$ – see front matter
PII: S0040-6031(01)00742-0

if we are going to exploit their catalytic potential fully we have to seek methods to stabilize the catalysts, i.e. the cells or organisms involved. For instance, optimising the conditions for productive degradation requires not only the maintenance of physical factors (such as temperature and pH) at ideal levels, but also ensuring that all nutrients are available in sufficient and well balanced concentrations ($[S] \gg K_S$). Only then can the supplied Gibbs energy and the metabolic network be optimally used to drive the essential life processes of maintenance, growth and multiplication. Every deviation from this state diminishes the growth rate or yield coefficients, although not necessarily the rate of substrate consumption. Such deviations will be reflected in changed heat fluxes and may be accompanied by changes in cell composition or exchanged material fluxes with the surroundings. However, the alterations in the composition of the cells and exchanged matter have to fulfill the First Law of Thermodynamics. Thus, comparison of the measured heat flux with the enthalpy balance may reveal previously undetected, unexpected events, providing a powerful means of checking the completeness of current physiological knowledge [1–3]. On the other hand, changes in the heat flux instantaneously reflect the metabolic changes [4,5].

Microbial responses can be unambiguously assigned to a certain stress or deficit by comparison of the growth pattern, i.e. material and energetic fluxes, of different continuous cultures, e.g. chemostat cultures grown under different conditions. However, the implementation of different steady states takes time and makes high demands on the stability of the calorimeter as well as the purity of the culture. Furthermore, we have to consider the possibility that cells may lose irreversibly their metabolic flexibility, if they are exposed for a long time to constant conditions. Use of transient-state cultivation techniques provides a way of avoiding these problems, in which all growing conditions are kept constant except for the concentration of one stress-exerting component. Therefore, the aims of our study were to develop such a calorimetric system, to derive mathematical tools for describing the concentration gradient as well as the microbial responses and, finally, to correlate the results with current biochemical knowledge.

2. Experimental

2.1. Organisms and culture conditions

Variovorax paradoxus DMSZ 4065 and *Halmonas elongata* DSMZ 2581^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). The strains were stored on agar slants at 4 °C and cultivated every 2 weeks: *V. paradoxus* on a minimal medium plus 10 mM fructose and *H. elongata* on a complex medium. The minimal medium was composed of (g/l): NH_4Cl (0.944); K_2HPO_4 (0.348); KH_2PO_4 (0.270); $MgSO_4{\cdot}7H_2O$ (0.0712); $CaCl_2{\cdot}6H_2O$ (0.0055); $FeSO_4{\cdot}7H_2O$ (0.00498); $CuSO_4{\cdot}5H_2O$ (0.000785), $MnSO_4{\cdot}4H_2O$ (0.00081), $ZnSO_4{\cdot}7H_2O$ (0.00044); $NaMoO_4{\cdot}4H_2O$ (0.00025); while the *Halomonas* medium contained: NaCl (180); casamino acids (with vitamins) (7.5); proteose peptone (5.0); yeast extract (1.0) Na_3-citrate (3.0); $MgSO_4{\cdot}7H_2O$ (20); KH_2PO_4 (0.5); $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ (0.05); and agar (17.0). *V. paradoxus* pre-cultures were prepared at 30 °C in shaking flasks containing 200 ml minimal medium with 6–8 additions of 100 mg/l portions of sodium benzoate to reach a biomass concentration of about 0.3 g/l, and the *H. elongata* was grown at 25 °C in shaking flasks containing 200 ml low-salt medium. The concentrated medium for feeding *V. paradoxus* in the fermenter calorimeter (see Section 2.2) contained 0.944 g NH_4Cl/l and all salts (except K_2HPO_4 and KH_2PO_4) at concentrations 10 times higher than in the minimal medium. A solution of 170 g KH_2PO_4/l and 218 g K_2HPO_4/l served as a separate source of phosphate. The low- and the high-salt media used to generate a salt gradient in the experiments with *H. elongata* were derived from [6] and were composed of (g/l): glucose (10); NH_4Cl (6); KH_2PO_4 (1.1); $MgSO_4{\cdot}7H_2O$ (1.4); $FeSO_4{\cdot}7H_2O$ (0.005); Tris (0.255); and 10 μl silicone anti-foam emulsion M-30 (Serva, Heidelberg, Germany) with 30 and 250 g NaCl /l, respectively.

2.2. Cultivation

V. paradoxus and *H. elongata* were grown continuously on sodium benzoate and glucose, respectively, as the sole sources of carbon and energy in a fermenter calorimeter (Berghof, Enningen, Germany). The

calorimeter has been described in detail elsewhere [7] and could be described as an isothermal heat flux calorimeter with power compensation, following the nomenclature suggested by Hansen [8]. The experiments were carried out at 30 °C for *V. paradoxus* and 25 °C for *H. elongata*. The pH was kept constant at 7.0 by titration with 1 M HCl, when sodium benzoate was the carbon source, and 0.5 M NaOH in the case of glucose. The heat of neutralisation was calculated from the titrimetric consumption of alkali or acid using tabulated values [2]. The temperature of all feeds was adjusted to the reactor temperature before they entered the vessel. The calorimeter was aerated with 0.1 $m^3\ h^{-1}$ air (related to 101.325 kPa and 20 °C) and saturated with water at reactor temperature. The culture was agitated using a Rushton-turbine at a rate of 400 rpm, and the oxygen level was always greater than 30% saturation. Torque measurements were used to detect the baseline shifts of the heat flux, according to a method proposed by Menoud et al. [9].

2.3. Analyses

The culture broth was rapidly separated from the biomass by direct ultrafiltration of the efflux stream from the calorimeter [10]. Poly-3-hydroxybutyrate (PHB) was quantified by gas chromatographic analysis after hydrochloric acid propanolysis [11]. Ectoine was determined after extraction from freeze-dried cells using isocratic HPLC and a refractive index monitor as the detector, as described in detail previously [12]. Sodium benzoate concentrations were monitored by HPLC on a Nucleosil 100 PR 18 column (250 mm × 3 mm i.d., Knauer-Säulentechnik, Berlin Germany) with 70% acetonitrile in water containing 0.1% acetic acid anhydride as the eluant. For ammonium measurements, a photometric assay (Spectroquant, Merck, Darmstadt Germany) based on indophenol blue reaction was used. Salt concentrations were quantified by titrating the chloride ions with silver nitrate, and using potassium chromate as an indicator [13]. Glucose was indirectly determined using a commercially available enzyme kit from Boehringer (Mannheim, Germany), whereby glucose was first phosphorylated and then oxidised by NADP. The amount of NADPH formed in this reaction is stoichiometric with the amount of glucose. NADPH was measured spectrophotometrically at 334 nm. The composition of the dried air leaving the calorimeter was determined by the passage through infrared carbon dioxide and electrochemical oxygen analysers (Ultramat 23, Siemens, Karlsruhe, Germany).

3. Results and discussion

3.1. Consideration of mass and energy balances

As a rule, the steady states of a carbon substrate-limited chemostats (i.e. where the concentration of the actual carbon substrate is low and governs the growth rate) are reached, by definition, when any rates are constant. The efficiency of carbon conversion of most organic substrates [$C_{S1}H_{S2}O_{S3}$] is energetically limited [14] and all other stoichiometric coefficients depend on it. This means that the theoretical maximum yield coefficient (carbon metabolism-determined) $Y^{\max}_{X/S}$ cannot be reached [15]. The energy E_A needed for biomass ($C_{X1}H_{X2}O_{X3}N_{X4}$) synthesis must be generated by the biological combustion of the carbon substrate offered.

$$C_{S1}H_{S2}O_{S3} + E_A \overset{NH_3}{\rightarrow} Y^{\max}_{X/S} C_{X1}H_{X2}O_{X3}N_{X4} + (S1 - X1\, Y^{\max}_{X/S})CO_2 \quad (1)$$

$$C_{S1}H_{S2}O_{S3} \overset{+O_2}{\rightarrow} S1\, CO_2 + E_D \quad (2)$$

If we increase the concentration of any substrate, while keeping the level of all other medium components constant, it will eventually influence the growth rate when it surpasses a specific level. If, for instance, the carbon substrate is increased, another nutrient will eventually become growth-limiting, and if the concentration of any substance becomes osmotically significant, growth may be limited by dehydration of the cells. In addition, many substances are toxic above certain concentrations.

There are several consequences of these phenomena for the microorganisms. Firstly, if cells are unable to adapt to adverse circumstances, the life processes become unstable and the carbon substrate accumulates. Secondly, cells may stabilise their viability by adaptive mechanisms. During the so-called nutrient-limited growth excess carbon and energy may be wasted in the form of heat or carbon dioxide, by excretion of incompletely combusted carbon residues or by intracellular accumulation of metabolites

(frequently as polymers such as PHB) [16]. These intermediates of overflow metabolism are often called overflow metabolites. During osmotically influenced growth, microorganisms withstand reductions in water activity predominantly by synthesising or (taking up) compatible solutes as an osmotic counterweight [17]. In both the cases, the fluxes of energy and/or formation rates of such metabolites or compatible solutes depend on the concentration of the stress exerting substrate, at least within a certain range. The synthesis (or uptake) of compatible solutes or formation of overflow metabolites, $C_{CS1}H_{CS2}O_{CS3}N_{CS4}$, influences the growth yield coefficient according to the energy required for their synthesis, E_{CS}.

$$C_{S1}H_{S2}O_{S3} + E_{CS} \overset{+NH_3,O_2}{\rightarrow} Y^{max}_{CS/S} C_{CS1}H_{CS2}O_{CS3}N_{CS4} + (S1 - CS_1\, Y^{max}_{CS/S})CO_2 \quad (3)$$

The uptake may be considered as an energetically neutral or energy-consuming reaction. Above a certain level of stress, the ability of the cells to withstand the adverse factors by synthesising the overflow metabolites or compatible solutes is exhausted. If the compatible solutes or overflow metabolites are commercially interesting, then the point at which this occurs could be considered the economic optimum [18], because the formation rate of these products is then at a maximum. If the strength of stress increases further, growth will be limited by the uptake rate of the carbon substrate and a wash out will occur. Analysis of these rates and heat fluxes should be useful for determining E_{CS} and Y^{max}_{CS}, i.e. the energetic and material costs of the adaptation process. The heat flux reflects the instantaneous metabolic changes and the comparison of enthalpy balance with the measured heat flux provides information about the completeness of the analysis of the rate changes.

For such considerations, a special feeding system (Fig. 1) is required to gradually change the concentration of one substrate while keeping all the other growth conditions constant. In our experiments, chemostatic growth was initially established by maintaining a constant flow, F_2 (l h^{-1}), from the mixing flask with the low, initial concentration (S_0^{IN}, mol l^{-1}) of the substance to be varied. After reaching a steady state, as indicated by a constant heat flux, the concentration gradient was started by switching on a constant flow from the reservoir flask, designated F_1 (l h^{-1}) with the higher concentration (S^R, mol l^{-1}) of this substance. The concentration of all other nutrients was kept the same in both the flasks. The shape of the concentration gradient depends on the ratio of F_2 to F_1 [19]. The time course of the concentration of the substance varied in the calorimeter input (S^{IN}, mol l^{-1}) (Eqs. (4) and (5))

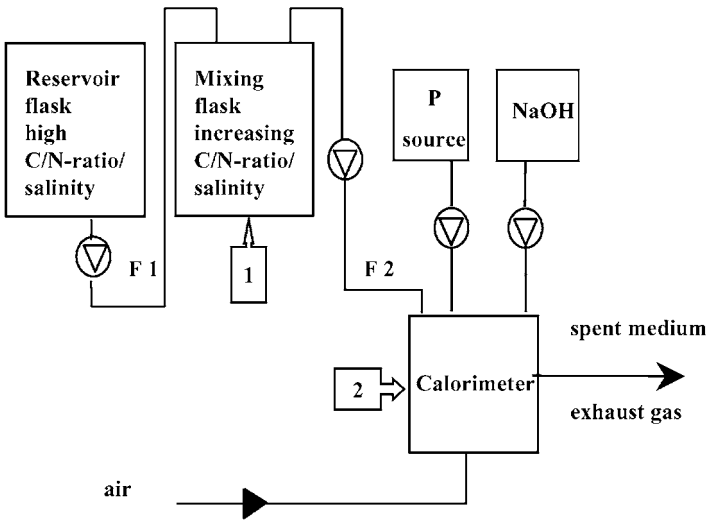

Fig. 1. Experimental apparatus used to generate concentration gradients at a constant dilution rate and constant supply of all other nutrients. F_1 and F_2 denote fluxes from the reservoir and mixing flasks, respectively. The time course of the concentration of the substance varied is described at point 1 by Eqs. (4) and (5), and at point 2 by Eqs. (6) and (7).

is given by integrating the respective differential balance equation of the mixing flask.

If $F_2{:}F_1 \neq 1$

$$S^{IN} = S^R - \left[S^R - S_0^{IN} \left(\frac{V_0 + (F_1 - F_2)t}{V_0} \right)^{[F_1/(F_2-F_1)]} \right] \quad (4)$$

If $F_2{:}F_1 = 1$

$$S^{IN} = S^R - \left[(S^R - S_0^{IN})\, e^{-F_1 t/V_0} \right] \quad (5)$$

where V_0 is the volume (l) of the mixing flask at time 0 and t the time (h).

This time course describes the changes in the strength of the stress exerted by substances that are consumed by the microorganisms. If we want to evaluate the strength of stress exerted by substances that do not participate in metabolism, then we have to consider the time course of the concentration in the calorimeter itself (S^C, mol l^{-1}). The analytical integration of the respective differential balance equations succeeded only for two ratios of $F_2{:}F_1$, (Eqs. (6) and (7)).

If $F_2{:}F_1 = 2$

$$S^C = \frac{(S^R - S_0^{IN})F_2}{2V_0 D}(Dt - 1) + S_0^{IN} + \left[\frac{(S^R - S_0^{IN})F_2}{2V_0 D} - S_0^{IN} + S_0^C \right] e^{-Dt} \quad (6)$$

or if $F_2{:}F_1 = 1$

$$S^C = S^R + (S_0^C - S^R)\, e^{-Dt} + \frac{DV_0}{DV_0 - F}(S^R - S_0^{IN})(e^{-Dt} - e^{-Ft/V_0}) \quad (7)$$

where D is the dilution rate (F_2/V_C), V_C the calorimeter content (l), S_0^C the substance concentration in the calorimeter at time 0 (mol l^{-1}).

To derive Eqs. (4)–(7), it was assumed that mixing was complete in the mixing flask as well as in the calorimeter, and that there was a negligible excess mixing volume. It was found from sterile experiments that both the assumptions are valid and that the heat of mixing can often be neglected (results not shown). If S_0^{IN} is chosen according to Eq. (8), then Eq. (6) describes a linear concentration gradient.

$$S_0^{IN} = \frac{2V_0 S_0^C + V_C S^R}{2V_0 + V_C} \quad (8)$$

The maximum possible concentration in the calorimeter (S^{max}), must be lower than S^R, depending on the ratio of the volumes of the mixing flask and the calorimeter (Eq. (9)).

$$S^{max} = \frac{2V_0 S^R + V_C S_0^C}{2V_0 + V_C} \quad (9)$$

There is a conflict between the requirement for high S^{max}, which is advantageous for investigations of the upper limit of osmo- or halo-adaptation, and a steep gradient, which helps to diminish the error caused by the baseline shifts of heat flux. High S^{max} requires a high $V_0{:}V_C$ ratio and high S_0^C, whereas a steep gradient needs these values to be low, according to Eq. (10).

$$\frac{dS^C}{dt} = \frac{(S^R - S_0^C)DV_C}{2V_0 + V_C} \quad (10)$$

The microbial adaptation process can be described using the elemental balances (of carbon, for instance, as shown in Eq. (11)) or, alternatively, the balances of enthalpy (Eq. (12)) or substrates (Eq. (13)) can be used. Eqs. (11) and (12) are restricted to linear evolutions of the rates and include the rate of formation of only one metabolite or compatible solute, respectively. However, the equations can simply be extended to describe more complex changes in the rates, or the rates of formation of additional substances, by considering the higher-order derivations of the rate evolutions and by inserting additional rate functions. Further equations can be generated by taking the curvature and skew of carbon dioxide release, or those of the heat flux, into consideration.

$$\frac{d\dot{n}_{CO_2}}{V_C\, dt} = -[CX\dot{r}_X + CY\dot{r}_Y - CS\dot{r}_S] \quad (11)$$

$$\frac{dP}{V_C\, dt} = -[\dot{r}_X(\Delta_C H_X - NX\Delta_C H_N) + \dot{r}_Y(\Delta_C H_Y - NY\Delta_C H_N) - \dot{r}_S \Delta_C H_S] \quad (12)$$

where CX, CY and CS are the molar fraction of carbon of the biomass, overflow metabolite (or compatible solute) and substrate, respectively, NX and NY the molar fraction of nitrogen of the biomass and overflow metabolite (or compatible solute), respectively; $\Delta_C H_X$, $\Delta_C H_Y$, $\Delta_C H_S$, and $\Delta_C H_N$ the combustion enthalpy (kJ mol^{-1}) of the biomass, overflow metabolite (or compatible solute), substrate and nitrogen source, respectively. r_X, $\dot{r}_X$, r_Y, $\dot{r}_Y$, r_S and $\dot{r}_S$ the rates (mol l^{-1} h^{-1})

and rate of change of rates (mol l^{-1} h^{-2}) of formation of biomass, overflow metabolites or compatible solutes and the consumption of substrate, respectively.

The connection between the measured time courses of any concentrations, Φ (mol l^{-1}), and the evolution of rates of formation $r^{\mathrm{Gen}} = r^{\mathrm{Gen}}(t)$ or consumption rates $r^{\mathrm{Con}} = r^{\mathrm{Con}}(t)$ of specific substances, is given by the respective balance for the calorimeter (Eq. (14))

$$\frac{\mathrm{d}\Phi}{\mathrm{d}t} = r^{\mathrm{Gen}} - r^{\mathrm{Con}} + D(\Phi_0 - \Phi) \tag{13}$$

The analytical integration of the differential Eq. (13), giving Eq. (14), can express the time course of any rates in terms of a polynomial of the degree n (Eq. (15)).

$$\Phi = \Phi^0 \mathrm{e}^{-Dt} - \left(\frac{\Delta a_0}{D} + \Phi_0\right)(\mathrm{e}^{-Dt} - 1) + \sum_{J=1}^{n} \frac{\Delta a_J}{D} \left((-1)^{(J+1)} \frac{J!}{D^J} \mathrm{e}^{-Dt} + \sum_{I=1}^{J+1} (-1)^{(I+1)} \times \frac{J! t^{(J+1-I)}}{(J+1-I)! D^{(I-1)}} \right) \tag{14}$$

$$r^{\mathrm{Gen}} - r^{\mathrm{Con}} = \sum_{J=0}^{n} \Delta a_J t^J \quad \text{with } \Delta a_J = a_J^{\mathrm{Gen}} - a_J^{\mathrm{Con}} \tag{15}$$

where Φ^0 is the concentration at time 0 (mol l^{-1}).

However, assuming a steady state (chemostat) at time 0, characterised by constant rates of $r^{\mathrm{Gen}} - r^{\mathrm{Con}} = \Delta a_0^{\mathrm{SS}}$, a simpler solution is obtained.

$$\Phi = \left(\frac{\Delta a_0^{\mathrm{SS}} - \Delta a_0}{D}\right) + \frac{\Delta a_0}{D} + \Phi_0 + \sum_{J=1}^{n} \frac{\Delta a_J}{D} \left((-1)^{(J+1)} \frac{J!}{D^J} \mathrm{e}^{-Dt} + \sum_{I=1}^{J+1} (-1)^{(I+1)} \times \frac{J! t^{(J+1-I)}}{(J+1-I)! D^{(I-1)}} \right) \tag{16}$$

In most cases investigated so far, a polynomial of the degree 1 was sufficient.

3.2. Adaptation to nitrogen shortage of *V. paradoxus* growing on sodium benzoate

To test the validity of the transient state technique, *V. paradoxus* was used, because its major response to nitrogen shortage is intracellular storage of excess carbon and reduction equivalents in PHB [18,20]. Fig. 2 shows the heat evolution and changes in concentration recorded for *V. paradoxus* growing in transient states on linearly increasing sodium benzoate concentrations, proceeding from a carbon substrate-limited chemostat. The constant heat flux (−608 mW), and rates of substrate consumption (72 mg l^{-1} h^{-1}), biomass formation (33.5 mg l^{-1} h^{-1}) and PHB con-

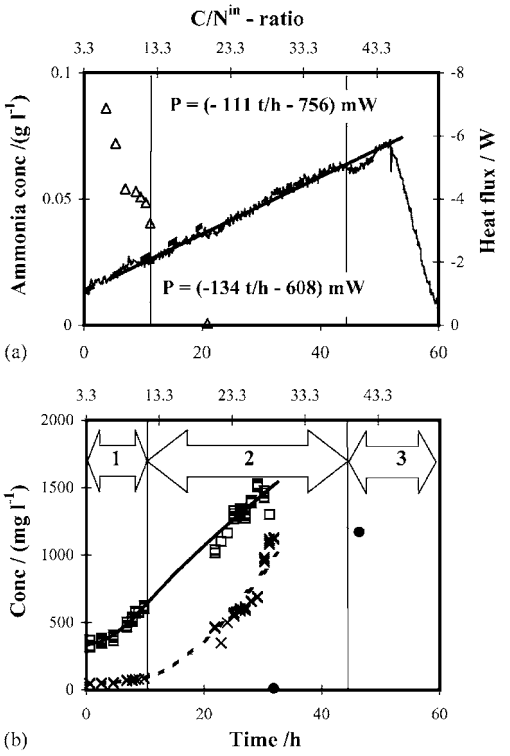

Fig. 2. Transient growth pattern of *V. paradoxus* growing on a linearly increasing sodium benzoate concentration as a function of time (primary abscissa) or C:N^{in} ratio (secondary abscissa), respectively. The gradient proceeds from chemostatic growth (dilution rate, 0.1 h^{-1}; input concentration, 0.72 g l^{-1} sodium benzoate). (a) Ammonia concentration (open triangles); heat flux (thin line); interpolation of heat flux during the first transient growth phase (dotted bold line); interpolation of heat flux during the second transient growth phase (bold line). (b) PHB-free cell dry mass (open rectangles); PHB (crosses); residual sodium benzoate (closed circles); course of biomass assuming a linear increase of biomass formation rate (phase 1, $r_X = (33.5 + 7.9t)$ mg l^{-1} h^{-1}; phase 2, $r_X = (112.5 + 3.5(t - 10))$ mg l^{-1} h^{-1}) (bold line); course of PHB concentration assuming a linear enhancement of the respective formation rate (phase 1, $r_{\mathrm{PHB}} = (4.5 + 1.12t)$ mg l^{-1} h^{-1}; phase 2, $r_{\mathrm{PHB}} = (16.3 + 6.3(t - 10))$ mg l^{-1} h^{-1}) (dotted bold line).

tent (4.5 mg l^{-1} h^{-1}) all indicate that steady state had been attained before the concentration of sodium benzoate began to change. Using tabulated combustion enthalpies for ammonia [2] and benzoic acid [21], assuming a formula of $C_4H_8O_2N_1$ [14] for PHB-free cells and applying Thornton's rule [22] and its improvement by Cordier et al. [23] for estimating the energy content of the cells and PHB, a heat flux of −513 mW can be calculated from the enthalpy balance, consistent with the experimental data. The biomass and PHB yield coefficients were 0.465 and 0.063 g g^{-1}, respectively. The first transient growth phase of the *V. paradoxus* was carbon substrate-limited. The best description of the microbial response to increasing sodium benzoate supply (17.7 mg l^{-1} h^{-2}) was obtained assuming the linear increases of biomass formation (7.9 mg l^{-1} h^{-2}) and PHB formation (1.12 mg l^{-1} h^{-2}). The accompanying enthalpy balance predicts a linear decrease of heat flux (−129 mW h^{-1}), in close agreement with the empirically obtained figure (−134 mW h^{-1}). The ratio of the evolution of the formation rates of PHB to biomass (0.141) is similar to the ratio of the rates under steady state conditions (0.134), as would be expected, assuming that metabolic fluxes are similar under both steady state and transient-state conditions. Major features of the second transient phase (starting at $t = 10$ h), i.e. nitrogen-limited growth, were linearly accelerated formation of PHB ($r_{PHB} = (16.3 + 6.3(t - 10))$ mg l^{-1} h^{-1}) and linearly delayed the formation of biomass ($r_X = (112.5 + 3.5(t - 10))$ mg l^{-1} h^{-1}). Both changed fluxes require a changed slope of heat flux (−109 mW h^{-1}) consistent with the data obtained (−111 mW h^{-1}). The third transient growth phase started as soon as the capacity of the cells to utilise further carbon and energy substrate was exhausted. The benzoate then accumulated and the cells were poisoned. Thus, the data appear to confirm (i) that the proposed model is able to describe the adaptation process and (ii) the response of *V. paradoxus* to nitrogen shortage consists predominantly of storing excess carbon and energy in the form of PHB.

3.3. Halo adaptation of H. elongata growing on glucose

To test the proposed method for evaluating changes induced by stress-exerting substances that do not participate in metabolism, the halo adaptation of *H. elongata* provides an excellent model. This is because the organism maintains osmotic equilibrium by adjusting the intracellular levels of just one major compatible solute [24], i.e. ectoine. Therefore, it is an ideal organism for thermodynamic analysis. In our experiments, *H. elongata* was the first grown chemostatically in the presence of 3% sodium chloride, with a constant heat flux (−2550 mW) and constant rates of dilution (0.071 h^{-1}), substrate consumption (710 mg l^{-1} h^{-1}), growth (374 mg l^{-1} h^{-1}), formation of ectoine (21 mg l^{-1} h^{-1}) and carbon dioxide (22.4 mmol h^{-1}) (data not shown).

The theoretically expected figures for the balances of enthalpy (−2523 mW) and carbon (18.0 mmol h^{-1}) are consistent with the experimental data, indicating that the flux analysis was complete. In contrast to the experiments involving stress exerted by nutrient shortages, adaptive processes are required as soon as the salt gradient starts. In accordance with the postulated hypotheses, two transient growth phases can be distinguished in *H. elongata* from the evolution of heat fluxes and CO_2 release (Fig. 3). As soon as the salt gradient started, the heat flux increased and the carbon dioxide formation rates decreased linearly until the NaCl content reached 14% NaCl ($t = 39$ h). The best description of this halo adaptation phase was obtained assuming a linear enhancement of ectoine formation (Eq. (17)) (Fig. 3) and a linear reduction of the biomass formation rate (Eq. (18)) (results not graphically shown).

$$r_E = (21 \pm 4)\,\text{mg}\,l^{-1}\,h^{-1} + (2.6 \pm 0.2)\,t\,\text{mg}\,l^{-1}\,h^{-2} \quad (17)$$

$$r_X = (374 \pm 7)\,\text{mg}\,l^{-1}\,h^{-1} - (1.56 \pm 0.04)\,t\,\text{mg}\,l^{-1}\,h^{-2} \quad (18)$$

An increase in the heat flux of 11 ± 3 mW h^{-1}, and a reduction in CO_2 production rate of 0.10± 0.02 mmol h^{-2} would be expected from this time course, which is consistent with the measured figures of 17 ± 3 mW h^{-1} and 0.13 ± 0.02 mmol h^{-2}, respectively. According to the Y_{ATP}-concept [25,26] and flux analysis, it can be predicted that the growth-associated synthesis of 1 mol ectoine required just 1 mol glucose and hardly any energy, irrespective

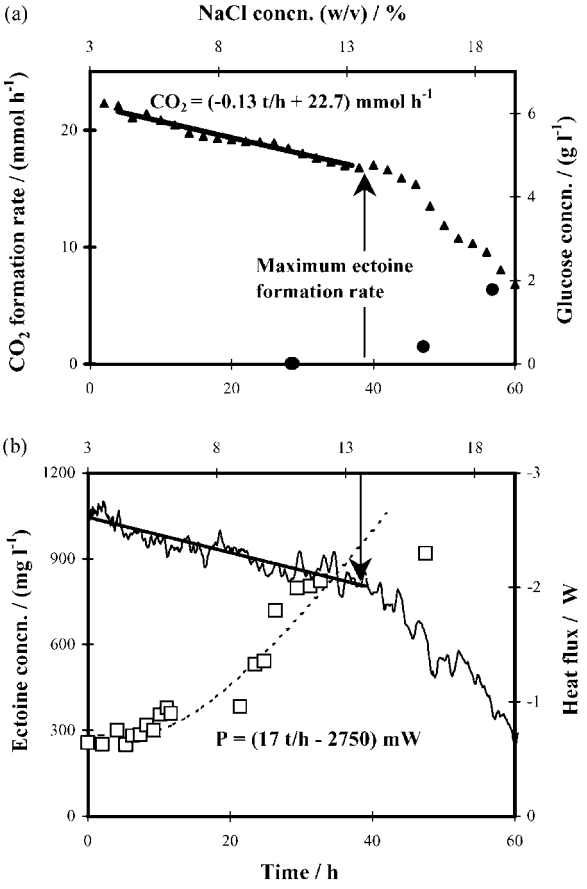

Fig. 3. Transient growth pattern of *H. elongata* growing on glucose, while sodium chloride concentration increases approximately linearly, as functions of time (primary abscissa) and NaCl concentration (secondary abscissa). The gradient proceeds from chemostatic growth (dilution rate, 0.071 h^{-1}; input concentration 10 g l^{-1} glucose; salt concentration, 30 g l^{-1}). (a) CO_2 formation rate (closed triangles); interpolation of CO_2 release (bold line); residual glucose concentration (closed circles). (b) Heat flux (thin line); interpolation of heat flux (bold line); course of ectoine content assuming a linear increase in its rate of formation (dotted line); measured ectoine concentration (open rectangles).

of the assimilation pathway involved, or whether the C4-precursor was supplied via the glyoxylate shunt or via C3 carboxylation [12]. At a certain salinity (14% NaCl, $t = 39$ h) the capacity of the cells to withstand further increases in the salinity was exhausted. Consequently, glucose accumulated in the medium and the rate of carbon dioxide release decreased and heat flux increased more rapidly.

4. Conclusions

The results indicate that the transient-state method is a powerful tool for quantifying and ascribing changes in material and energetic fluxes involved in microbial responses to specific deviations from ideal conditions. The method is based on the solution of functions describing changes in the key fluxes. Because any flux evolution may be expressed by a polynomial equation, the method should be generally valid. However, since the accuracy of both heat flux measurement and off-line analysis is limited, the method is restricted to main carbon-fluxes, and the rate evolutions are defined purely by linear or maximum quadratic polynomials. A simple error assessment shows that the results of the transient-state method are more reliable than the results obtained from the comparison of different steady states, and a more sensitive calorimeter could increase the accuracy of the method significantly. However, rapid developments in the design of bench scale calorimeters [5,27,28], miniaturised "nanocalorimeters" and integrated circuit calorimeters [29,30] and in the on-line monitoring of biomass and substrates are underway. These developments promise the extension of the developed method to more subtle environmental changes and metabolic responses in the near future.

Acknowledgements

The authors wish to thank Dr. Ackermann and Ilona Skinfill Rivera (Sächsisches Institut für Angewandte Biotechnologie e.V.) for PHB analysis and Falk Tätzner for his technical assistance.

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