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Use of reaction calorimetry to monitor and control microbial cultures producing industrially relevant secondary metabolites[1]

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Abstract

In this article, bench-scale heat flux calorimetry is applied to monitor complex microbial systems producing secondary metabolites and having a rheologically complex behavior. With such systems, biological metabolic activity can only be measured accurately if calorimetry is improved by on-line correction for stirring power variations using torque measurement.

First, a successful application to the production of the antibiotic erythromycin by *Saccharopolyspora erythraea* is presented. During a batch-culture study, it was shown that heat-flux calorimetry can indicate the two main phases of the process, the exact moment of any substrate depletion, and the nature of the depleted substrate. A fed-batch strategy was set up to optimize erythromycin production. Cultures controlled by calorimetry with pulsed addition of the N-source during the trophophase and of the C-source during the idiophase allowed a higher productivity to be reached.

Second, an application of heat-flux calorimetry to the production of a bioinsecticide by the sporulating bacterium *Bacillus sphaericus* is presented. This project aims to understand, model and control the factors that effect growth, sporulation and insecticide production in a chemically defined medium. A batch-culture study has shown that calorimetry can be used to monitor the different phases of the process (growth, sporulation) and the different substrate depletions. The use of a control strategy to optimize production of the insecticidal protein is now under investigation. © 1998 Elsevier Science B.V.

Keywords: *Bacillus sphaericus*; Bench-scale calorimetry; Control; Monitoring; *Saccharopolyspora erythraea*

1. Introduction

It is well known that heat dissipation is a universal feature of living systems [1] and that the measurement of heat-exchange rates can serve as a powerful indicator of the nature of an on-going biological process [2]. Because there are only a few probes that allow on-line monitoring of process variables and because calorimetry can be used at the industrial scale without sophisticated equipment [3], calorimetry has an obvious potential that has rarely been exploited. With the advent of bench-scale heat-flux calorimetry, application of this technique at the laboratory scale has increased during the last 15 years and is reviewed in Ref. [4]. In this contribution, bench-scale calorimetry is applied to monitor complex microbial systems producing secondary metabolites and having a rheologically complex behavior. For both applications

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presented below, heat flux had to be corrected for stirring power variations, as described earlier in Ref. [5], to get an accurate measurement of biological metabolic activity.

In the first example [6], bench-scale calorimetry was applied for on-line monitoring and control of bioprocesses with *Saccharopolyspora erythraea* as a model microorganism. This filamentous bacterium is chemoheterotrophic and an oligate aerobe. Its mycelial structure implies a growth in pellets even in well-agitated submerged cultures [7]. It grows on complex media containing starch [8] or oils and molasses [9] and on semi-defined media [8] and produces erythromycin A. This major antibiotic is known for its ability to inhibit protein synthesis of prokaryotic microorganisms [10] and is mainly used against pulmonary infections.

In a second example, the production of a bioinsecticide was investigated calorimetrically. For several decades, interest in biological alternatives to chemical pesticides has greatly increased. Among the alternatives, *Bacillus sphaericus*, a spore-forming, chemoheterotrophic and aerobic bacterium, has been shown to possess potent larvicidal activity against several species of mosquito, including those species that transmit malaria and filaria [11]. Active strains of *B. sphaericus* are known to produce proteinaceous, mosquito-larvicidal factors (MLF) at the onset of sporogenesis [12]. Economic cultivation of *B. sphaericus* is mainly hampered by its inability to use simple and inexpensive C-sources [13]. Proteinaceous complex media have been used for large-scale cultivation, but often the result is poor biomass and product yields [14,15]. To better understand factors that affect growth, sporogenesis and MLF production, some studies have been made with a defined medium but data are still incomplete [16–18]. This work used improved bench-scale calorimetry as a tool to monitor on-line cultures of *B. sphaericus* to produce data necessary for understanding and modeling this process.

2. Experimental

Materials and methods for erythromycin production by *S. erythraea* have already been published [6] and are not reported here.

2.1. Microorganism and medium

The *B. sphaericus* 1593M strain used in this study was obtained from Dr. K. Jayaraman (Center for Biotechnology, Anna University, Madras, India). It was grown in a defined medium [13], as modified by Suresh [16]. The composition was (g/l): Na_2HPO_4 (2.8), KH_2PO_4 (1.12), $MgSO_4{\cdot}7H_2O$ (0.1), $CaCl_2{\cdot}2H_2O$ (0.01), $MnCl_2{\cdot}4H_2O$ (0.004), $FeSO_4{\cdot}7H_2O$ (0.003), thiamine (0.03), biotin (0.003) and PPG 2000 (antifoam) at 0.1% v/v. C-sources were acetate ($NaCH_3COOH{\cdot}3H_2O$) and glutamate ($NaC_5H_9O_4N{\cdot}H_2O$). An additional N-source was ammonium sulfate. All the chemicals were from Fluka (Switzerland).

2.2. Culture conditions

The strain was maintained on agar slants at 4°C. For inoculum preparation, the strain was activated in a shake flask (SF) of nutrient broth medium at 30°C, transferred during the exponential phase into a second SF containing batch-culture medium, centrifuged during the exponential phase, and finally inoculated into the reactor (10% v/v). The temperature was maintained at 30°C, pH at 7 and stirring speed at 600 or 1000 rpm. Air was added at a constant rate. Dissolved oxygen concentration was maintained between 30 and 100% of air saturation.

2.3. Experimental set-up

The reactor was a bench-scale reaction calorimeter RC1 (Mettler–Toledo, Switzerland) supplied with all the instruments needed to have well-defined culture conditions and accurate heat-flux measurement (Fig. 1). Its structure and operating principle have been described in Ref. [2]. Exhaust-gas composition was measured by a paramagnetic analyzer for O_2 (540 A, Servomex®, Bioengineering, Switzerland) and by an infrared analyzer for CO_2 (Binos 100, Bioengineering, Switzerland). A torque measuring device (TG-02, Vibrometer, Switzerland) was used to correct the heat-flux for stirring-power variations as described previously [5]. A data acquisition device (NB-Mio16X board, LabVIEW software, National Instrument, USA; with Macintosh IIci, Apple, USA) was used to store all the on-line measured signals (Fig. 1).

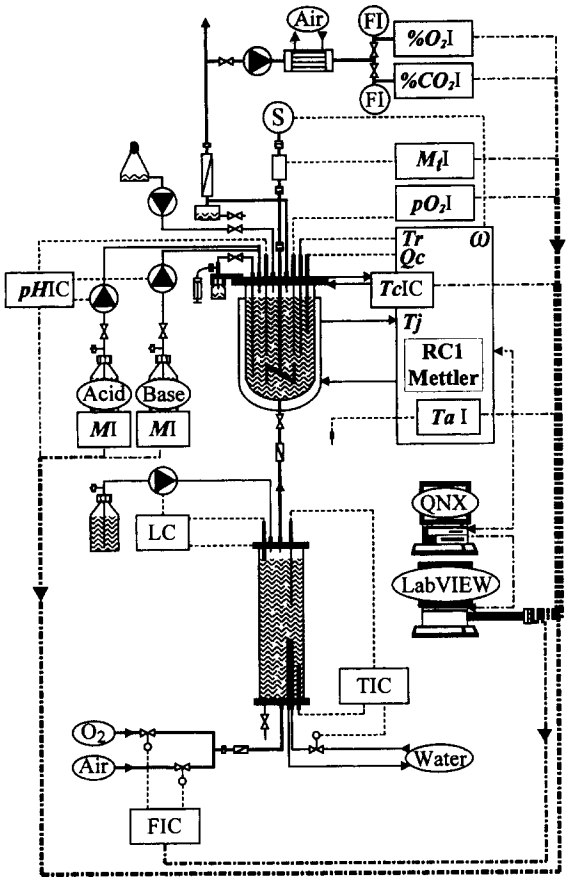

Fig. 1. Experimental set-up. Capital letters: T=temperature, %=volume fraction, Mt=torque, M=mass, Qc=calibration heat, ω=stirring speed, I=indicator, C=controller, F=flow, L=level and S=stirrer. Subscripts for T: r=reactor, j=jacket, a=ambient and c=cover. Solid lines represent tubing for the liquid or gas and dotted lines the electrical wires. Italic symbols in the figure represent parameters measured on-line.

2.4. Off-line analysis

Biomass concentration was measured by optical density at 600 nm and by dry weight after filtration with 0.2 μm-pore membrane-filters (Supor®-200, GelmanSciences, USA) and overnight drying at 105°C. Sporogenesis was followed by counting cells, sporangia and refractile spores under the microscope (400× magnification, Axiolab, Zeiss, Germany) in a counting chamber (Thoma, Hawksley, England). Acetate, glutamate and ammonium residual concentrations were measured with enzymatic reaction kits (Boehringer Mannheim, Switzerland).

The mosquitocidal product was quantified by a toxicity bio-assay on larvae of *Culex quinquefasciatus* at the III instar stage of development. Protein estimation was carried out using the method of Lowry et al. [19]. Toxicity is expressed as the lethal concentration for 50% of the population (LC_{50}) in nanograms of total protein per milliliters.

3. Results and discussion

3.1. Heat signal correction by stirring torque measurement

Menoud et al. [5] have shown, with batch cultures of *S. erythraea*, how heat-flux variation due to foaming problems can be corrected from torque measurements. In Fig. 2, the effect of stirring-power variations on the measurement of heat-production rate can be seen for a batch culture of *B. sphaericus*. In this case, despite the lack of foaming, the changes in stirring power would lead to an error of as much as 1 W if not taken into account. In the two previous cases, clear media were used for the cultures.

Figs. 3 and 4 show the results of a batch culture of *S. erythraea* on a complex medium containing starch. In these figures, the raw heat-production rate (see Fig. 3) appears to be completely different from the profile of the corrected rate (see Fig. 4). The corrected-rate profile is comparable to the profile of dissolved-oxygen concentration. These results show that torque measurement allows calorimetry to be used in highly heterogeneous systems with particulate media and that the torque measurement can eliminate the interference of many events such as changes of viscosity, aeration, stirring speed and volume. All further calorimetric curves presented in this paper have been corrected for stirring-power variations.

3.2. Application to erythromycin production

Figs. 5 and 6 show the profiles of a typical batch culture of *S. erythraea* on a semi-defined medium containing glucose (50 g/l), ammonium nitrate (2.19 g/l), an extract of cotton-seed flour (About 72% of cotton-seed flour mass is insoluble in water. After extraction of 30 g/l, only 8.4 g/l of complex

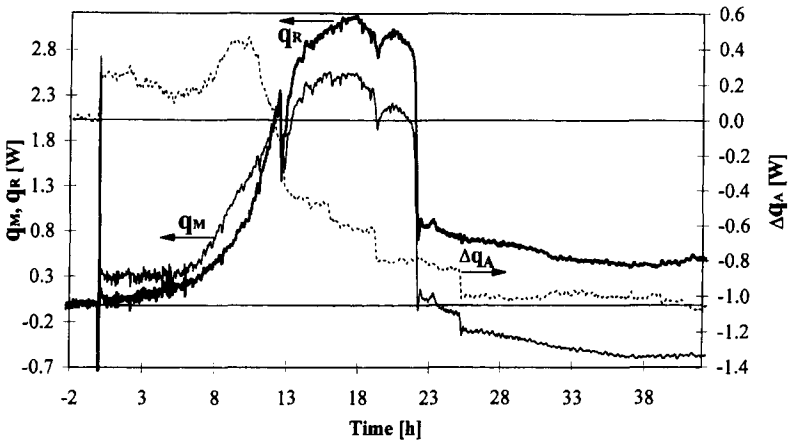

Fig. 2. Measured heat-dissipation rate (q_M), agitation-power variation (Δq_A), and corrected heat-production rate (q_R) during a batch culture of *B. sphaericus* in a defined medium.

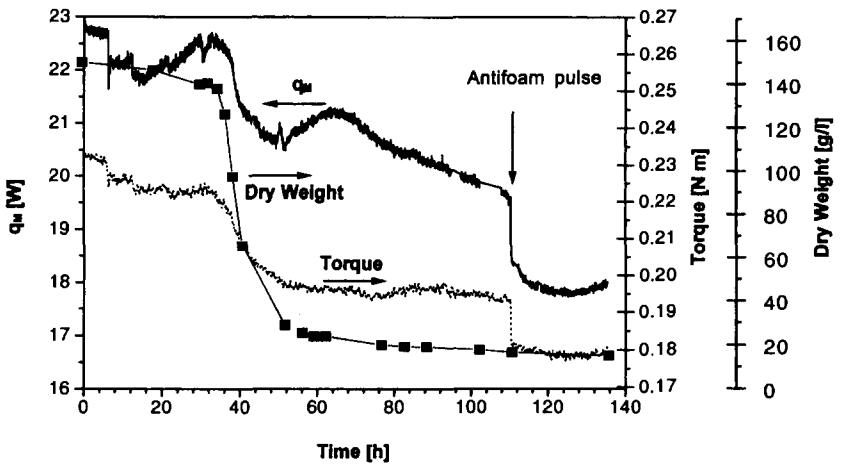

Fig. 3. Measured heat-dissipation rate (q_M), torque and dry weight during a batch culture of *S. erythraea* on a complex and heterogeneous medium containing starch [6].

substrate are effectively added to the culture medium) and some salts [6]. Concentration profiles of the substrates and products are presented in Fig. 5, where the curve connecting the markers for ammonium (triangles) represents an on-line measurement based on the consumption of NaOH needed to neutralize protons liberated by ammonia uptake. Fig. 6 presents the heat-production rate and shows a set of characteristic perturbations which were found to be quantitatively reproducible in all the other calorimetric profiles (not shown) and which can be used to identify particular metabolic events in the cultures.

The first event was a drop in the heat-production rate of ca. 1.4±0.14 W (labeled ⓪) that always occurred at the same time during the trophophase and is probably due to depletion of an unidentified substrate in the cotton-seed flour extract. Perturbations ② and ③, both consist of a small peak immediately followed by a period of high heat generation and can be clearly attributed to the depletion of N-sources, i.e. ammonium and nitrate respectively (Fig. 5). The high heat-production rate is most probably due to the tendency of microbial energy metabolism to uncouple when the microorganisms grow under excess energy

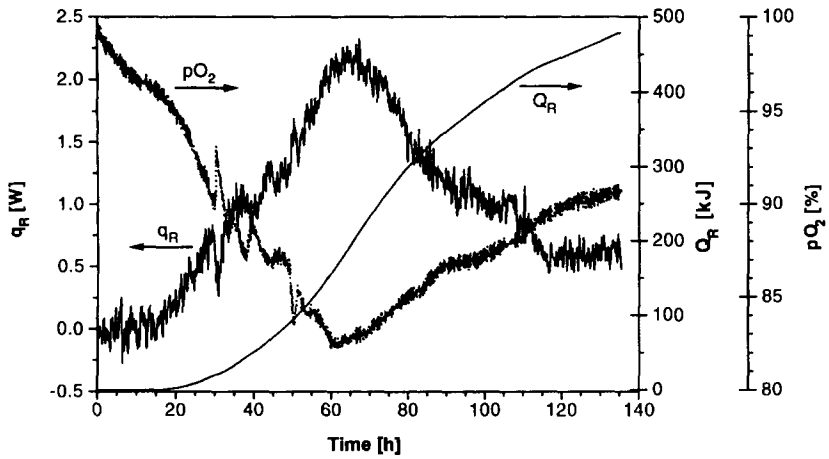

Fig. 4. Heat-production rate (q_R), dissolved-oxygen concentration (pO_2) and cumulated heat (Q_R) during the same batch culture of *S. erythraea* as Fig. 3. q_R is the result of the correction of q_M for base-line and torque variations and represents the actual heat produced by the microorganisms [6].

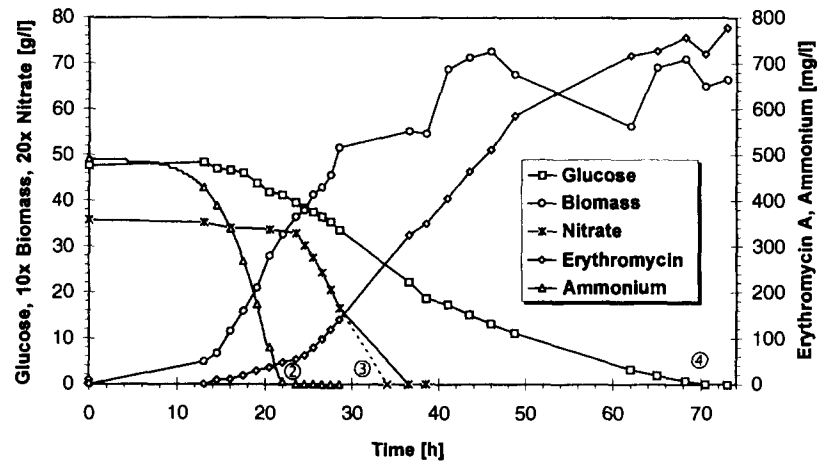

Fig. 5. Glucose-, biomass-, nitrate-, erythromycin-A- and ammonium-concentration profiles during a batch culture of *S. erythraea* on a semi-defined medium containing 50 g/l glucose and 2.19 g/l ammonium nitrate initial concentrations. ② indicates ammonium depletion, ③ nitrate depletion and ④ glucose depletion [6].

conditions [20]. The last event (labeled ④ is characterized by a sharp drop in the heat-production rate and can be clearly attributed to the depletion of glucose.

Fig. 6 shows that these events can also be detected by measurement of dissolved-oxygen concentration (pO_2) but only in a qualitative way. Indeed, this measurement depends on gas–liquid mass transfer of the air into the culture bulk. As the mass-transfer coefficient (k_La) can vary a lot during the cultures and as there is no way to monitor it, pO_2 measurement cannot be used to quantitatively monitor or control a culture. An illustration of that point can be seen in Fig. 6. A perturbation in the pO_2 profile occurred after 52 h, before the glucose depletion ④. This perturbation is not present on the power profile and is not linked to any substrate depletion and is, most probably, due to a change in the mass-transfer coefficient.

In this batch culture, erythromycin production began at the onset of the trophophase and continued up to the depletion of glucose. As already shown [8], in cultures grown in synthetic media, erythromycin is

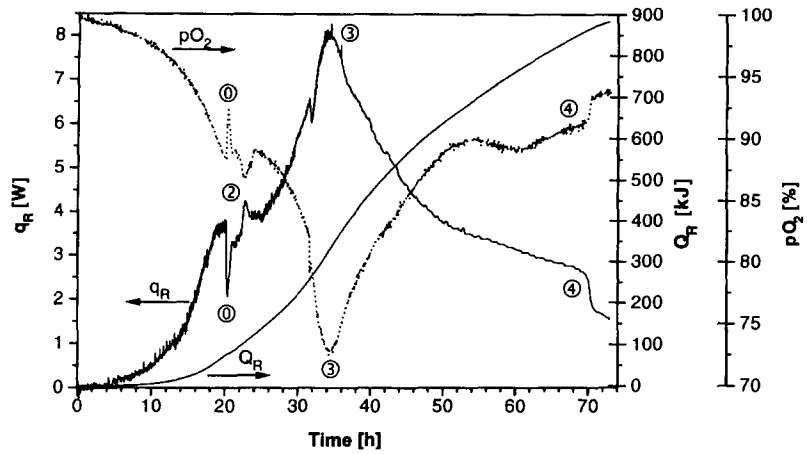

Fig. 6. Heat-production rate (q_R), dissolved-oxygen concentration (pO_2) and cumulated heat (Q_R) profiles during the same batch culture of *S. erythraea* as Fig. 5. ⓪ indicates a characteristic perturbation, ② ammonium depletion, ③ nitrate depletion and ④ glucose depletion [6].

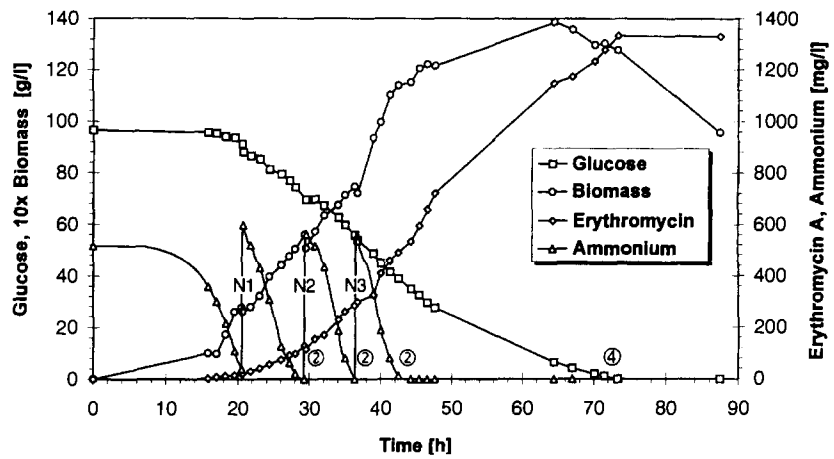

Fig. 7. Glucose-, biomass-, erythromycin-A- and ammonium-concentration profiles during a controlled fed-batch culture of *S. erythraea* on a semi-defined medium containing 100 g/l glucose and 0.5 g/l ammonium initial concentrations. Ammonium depletion was detected by the heat production signal shown in Fig. 8 and was compensated by three ammonium pulses (N1 to N3). ② indicates ammonium depletion and ④ glucose depletion [6].

not only produced during the idiophase but also during the trophophase. A set of experiments (not shown) at different initial concentrations of ammonium sulfate and ammonium nitrate showed that, with increased initial concentration of these two N-sources, the production yield of erythromycin during the trophophase was significantly lower due to an inhibition of biosynthesis by the N-sources [21,22]. The erythromycin yield during the idiophase was independent of the N-source concentration. These results show that productivity could be improved with a fed-batch culture by adding ammonium salts during trophophase to reach a high cell density with a maximum erythromycin production and then adding glucose to extend the idiophase as long as possible.

Such a fed-batch culture is presented in Figs. 7 and 8. It was started with an initial ammonium concentration of 0.5 g/l but with a high glucose concentration of 100 g/l. As can be seen in Fig. 8, after 30 and 42 h, the heat-rate profile show two similar perturbations that correspond to ammonium depletion (② in Fig. 7). Based on the characteristic shape of the heat-production rate described earlier (② in Figs. 6 and 8), ammonium was automatically restored to its initial

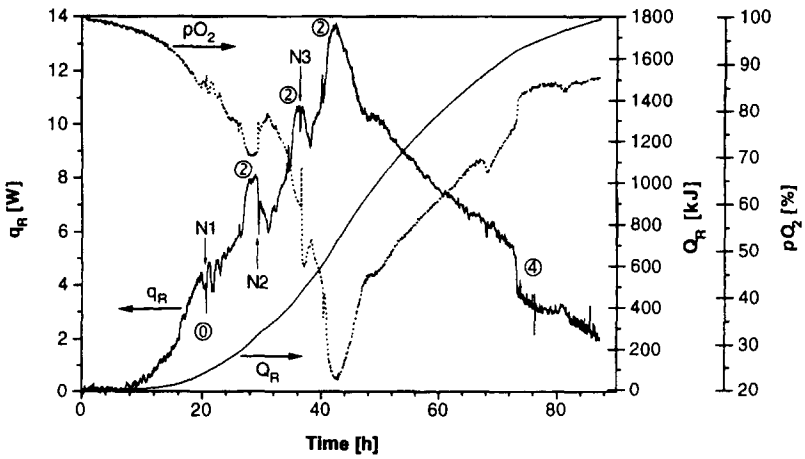

Fig. 8. Heat-production rate (q_R), cumulated heat (Q_R) and dissolved-oxygen concentration (pO_2) profile during a controlled fed-batch culture of *S. erythraea*. Same culture as Fig. 7. ⓪ indicates a characteristic perturbation, ② ammonium depletion, and ④ glucose depletion [6].

concentration by a pulse (N2 and N3 in Fig. 8), allowing growth to resume. After 20.5 h, the ammonium depletion occurs at the same time as the perturbation ⓪ described earlier (Fig. 8). In that case, the pulse (N1 in Fig. 7) was made a little before ammonium depletion because of the unusual shape of the heat-production rate profile. Three pulses were added in this way during this batch culture and after the last ammonium depletion; idiophase took place up to glucose depletion shown by a sharp drop of the heat-production rate (④ in Fig. 8). This particular shape of the calorimetric signal has been used in another experiment to extend the erythromycin production phase by addition of glucose pulse [4]. Overall performance of such a fed-batch was much better than that of a batch culture. The yield of erythromycin to substrate was increased by 15%, the production rate of erythromycin by 50% and the final erythromycin concentration by 80%. This type of fed-batch culture is expected to be especially attractive because it allows a high density of microorganism without inhibiting the erythromycin production and also because it avoids catabolic repression problems caused by too high initial glucose concentrations.

3.3. Application to bioinsecticide production

In this section, the results from two batch cultures are presented. *B. sphaericus* was grown on a defined medium containing 7.23 g/l of acetate and 0.58 g/l of glutamate and at two different concentrations of ammonium sulfate. These experiments were performed to correlate the heat-production rate to metabolic events during the culture, to determine coefficients of growth stoichiometry and kinetics and to try to close carbon, degree-of-reduction and enthalpy balances to check that all important compounds engaged were measured. The exact calculation procedure has been described previously [23]. Figs. 9 and 10 show the profiles of the substrates, products and heat-production rate for these batch cultures containing 0.5 g/l and 2 g/l of ammonium sulfate, respectively. As described in the previous section for *S. erythraea*, Figs. 9 and 10 clearly show that calorimetry can be used to monitor pertinent events of a culture of *B. sphaericus*.

In Fig. 9, the following set of characteristic perturbations can be seen. After 10 h, the culture reached the perturbation ①, a sharp drop in the heat-production rate that can be clearly attributed to the depletion of glutamate. Until that time, glutamate and acetate seem to have been consumed together, as the ammonium content only decreased moderately. This shows that glutamate is the preferred N-source. After this perturbation, heat production resumed and even continued to rise along with biomass concentration up to the perturbation ②, where heat-production rate decreased. During the same period acetate and ammonium were consumed; thus, a second growth phase on acetate as sole C-source took place between ① and ②. Looking

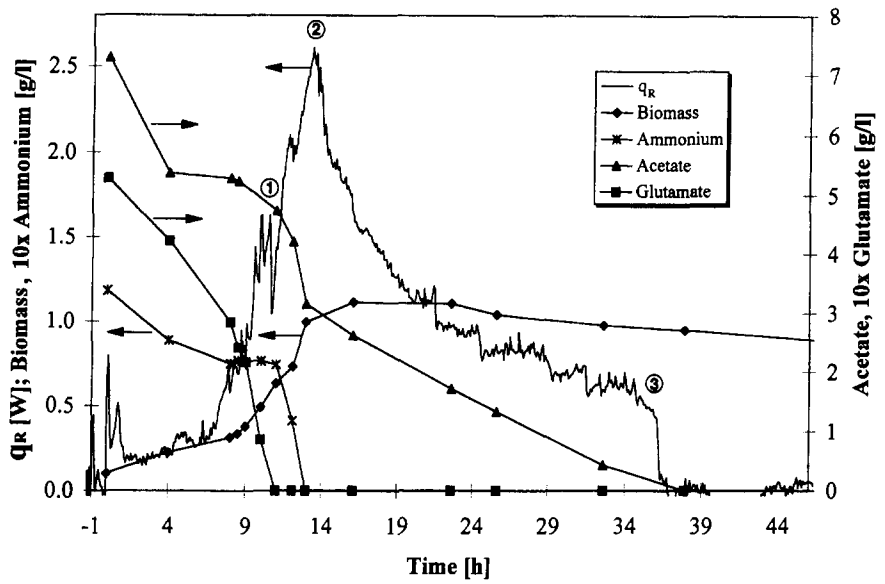

Fig. 9. Heat-production rate (q_R) and glutamate-, acetate-, ammonium- and biomass-concentration profile during a batch culture of *B. sphaericus* on a defined medium containing initial concentrations of 7.23 g/l acetate, 0.58 g/l glutamate and 0.5 g/l ammonium sulfate. Stirrer speed was 1000 rpm, initial volume, 1.7 l and aeration 0.7 vvm. ① indicates glutamate depletion, ② ammonium depletion and ③ acetate depletion.

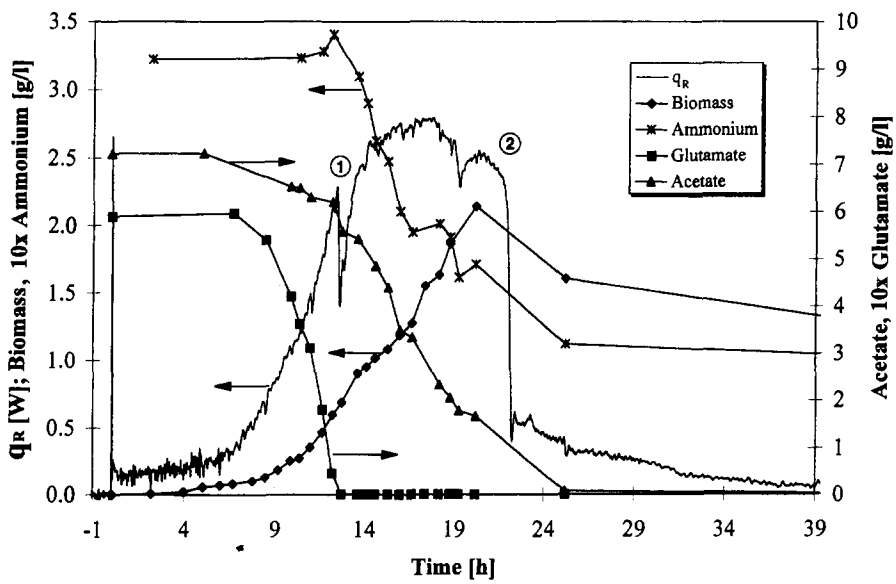

Fig. 10. Heat-production rate (q_R) and glutamate-, acetate-, ammonium- and biomass-concentration profile during a batch culture of *B. sphaericus* on a defined medium containing initial concentrations of 7.23 g/l acetate, 0.58 g/l glutamate and 2 g/l ammonium sulfate. Stirrer speed was 600 rpm, initial volume 1.8 l and aeration 0.5 vvm. ① indicates glutamate depletion and ② acetate depletion.

at the ammonium concentration profile, the second perturbation can clearly be attributed to the depletion of the second N-source. The shape of this second perturbation is reminiscent of the one described above for N-source depletions during cultures of *S. erythraea*. The last perturbation (③), a sharp drop in the heat-production rate, clearly has the shape of an energy source depletion as already described for *S. erythraea*. It corresponds exactly to the measured depletion of acetate. The calorimetric signal shows a high but decreasing heat-production rate between perturbations ② and ③, due mainly to the uncoupled oxidation of acetate. Since acetate concentration decreased linearly during this period, meaning that the consumption rate was constant, the heat-production rate due to this reaction is expected to be constant. A reduction in the heat-production rate indicates that some other reactions took place during that period.

Indeed, microscopic observations made at the second perturbation showed that 40% of the cells were already in the sporangium stage, meaning that the depletion of glutamate provoked a partial sporogenesis in spite of the availability of another C- and N-source. Other observations during the sporogenesis phase showed that the ratio of spores to cells increased but reached only 30% at the end of the culture. As a good sporogenesis is expected to reach a ratio of 100% of spores, this batch culture was found to give a weak sporogenesis. The reason for such a weak sporogenesis is unclear but could be attributed to a bad preparation of the inoculum. It has been reported for other sporulating microorganisms that synchronous cultures and sporogenesis can be obtained by 4–5 sequential transfers of exponentially growing culture to fresh medium [24]. The results reported in this chapter show that calorimetry can also be used to monitor the quality of sporogenesis, and to a certain extent the quality of the product.

To determine growth parameters, another experiment was performed in the same conditions, except that the ammonium sulfate concentration was increased to 2 g/l to extend the period of growth on acetate. As can be seen in Fig. 10, the first phase of the growth (up to perturbation ①) was similar to the one in Fig. 9, but the second phase was completely different. In this case, as there was enough nitrogen, the second growth phase lasted up to the depletion of acetate, clearly shown by the sharp drop of the heat production rate ②. Thereafter, this rate decreased slowly to zero, showing that microorganisms have a low metabolic activity during sporogenesis due to the breakdown of proteins and storage compounds that were produced during the vegetative phase [24].

The heat-production rate between perturbation ① and ② is not proportional to the biomass concentration any more. This could be explained by the fact that during this period, the culture is heterogeneous: a fraction of the population has entered sporogenesis (following glutamate depletion) and the other fraction is still growing on acetate. The biomass-concentration profile also showed a clear transition from one growth phase into another near the first perturbation. Growth rates were determined from these results: for growth on glutamate and acetate, the rate was $0.35\pm0.004\ h^{-1}$ and for the second phase, $0.2\pm0.002\ h^{-1}$. This second rate is smaller than the one measured during batch culture on acetate alone ($0.25\pm0.003\ h^{-1}$), data not shown). This could be explained by the decrease of the biomass dry weight during sporogenesis (see Fig. 9) and, perhaps, also to an adaptation time needed to grow under the new conditions. Such adaptation has previously been reported for yeast [25].

This work mainly focused on calorimetry, but many other parameters are also important and have been simultaneously monitored in order to fully characterize the process. From exhaust-gas composition measurement, balances have been made on carbon, degree-of-reduction and enthalpy of combustion as described in Refs. [23,26,27]. Recovery ratios (outputs/inputs) are 0.93, 1.00 and 0.98, respectively; hence showing a good characterization of the system and a good accuracy of the data. However, even more attention should be paid to the determination of the substrate concentrations to determine the exact stoichiometry of such a growth on mixed substrates. It can be noted also that sporulation was much better in the second batch (80%) and that the LC_{50} measured in this case was ca. 2 ng/ml, expressing a toxicity comparable to that reported previously for similar culture conditions [16,17].

4. Conclusions

Bench-scale calorimetry may be used as a reliable and quantitative indicator of the metabolic state of a

microbial culture. This technique can be used to monitor growth and stationary phases where secondary metabolites may be produced. In the second case, calorimetry can give information on the quantity and quality of these products.

A prerequisite for using calorimetry as a quantitative technique is to correct the measured heat-dissipation rate for stirring power variations that often occur, especially during transient cultures. With this on-line correction, calorimetry may be used safely to control biological processes using fed-batch strategies. On the contrary, it is shown that pO_2 measurement can only be used to monitor qualitatively and not control biological aerobic processes.

Feed-back control of fed-batch cultures of *S. erythraea* with calorimetry allowed productivity of erythromycin A to be significantly increased. Monitoring batch cultures of *B. sphaericus* allowed a better qualitative and quantitative understanding of the growth stoichiometry and kinetics and of the sporulation phase. Further work with this strain will be performed to evaluate the need for more sophisticated culture strategies.

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