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Application of batch-calorimetry for the investigation of microbial activity

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Abstract

Calorimetric methods have been used for investigation of the microbial activity. Investigations of a microbial culture were carried out with batch calorimeters to find a procedure for reproducible measurements and to get information about the physiological state of the microorganisms. The model organism was *Paracoccus denitrificans*. As a result of this work it was possible to reproduce the calorimetric experiments and provide a standard procedure for further investigations of microbial systems. The experiments showed a two-fold limitation (oxygen, glucose), which was clearly identified by calorimetric measurements under variation of the oxygen supply in combination with additional analysis (glucose, nitrate). Based on an equation for the metabolism of the cell culture the heat exchanged has been calculated and compared with the heat detected during the cultivation.

Keywords: Calorimetry; Energy balance; Growth equation; Microbial activity; *Paracoccus denitrificans*

1. Introduction

The growth behaviour of microorganisms is interesting for several reasons. It is important for the understanding of the interaction between chemical substances and the environment, for the investigation of antibiotic drugs or for optimisation of production strains in biotechnology to mention only three of the numerous applications. In many interesting cases the growth behaviour cannot be investigated by the methods commonly used in microbiology, like photometric methods, since the special requirements of the methods are not fulfilled, signals are generated from the not living part of the system also and/or the reproducibility of the measured signal–time behaviour is not practicable. Therefore a more unspecific approach is required, which should be widely applicable.

Living systems are inseparably combined with the proceeding of chemical reactions. They are for microbial systems the indispensable source, e.g. for energy supply, for the growth process and for the biomass production. All these reactions take place at constant pressure and therefore they are connected with an exo- or endothermic change of enthalpy. The sum of the enthalpy changes of all reactions can be exchanged in the form of heat between the living matter and its environment [1]. The generated heat or thermal power for the running processes is completely non-specific, but also a universal measure. The thermal effect is correlated to the living part of the system mainly.

The method for the detection of exchanged heat is calorimetry. Calorimetry has the advantages of being unspecific, non-invasive and insensitive to the electrochemical and optical properties of the investigated system. Regarding these features calorimetry appears well suited for the online monitoring of bioprocesses [1] studying the growth process of microbial cultures or for the detection of biological key components [2,3]. Various groups have done scientific work on the investigation of microbial growth with calorimeters with focus on different aspects, for instance: influence of environmental conditions [4–6], degradation of harmful substances [7,8], pharmaceutical aspects and effects of biocides [9–11] and/or biotechnological production processes [12,13]. The activity of the living cells within a microbial culture is characterised by the produced thermal power and therefore measurable with different calorimetric methods.

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Batch-calorimeters as well as flow-calorimeters have been applied. Their advantages and disadvantages have been discussed [14,15]. Furthermore, different volume scales of cell cultures are under observation [12,13,16].

Calorimetric measurements allow to draw up energy balances for the running chemical reactions during the growth process [17]. The slopes of the power–time and heat–time curves permit conclusions about the growth rate, the occurrence of limiting conditions and they give hints about the formation of metabolites which can be degraded subsequently [7]. The combination of the calorimetric results with more specific analytical investigations for the carbon sources, electron acceptors and metabolites provide a systematic description of the metabolism for the investigated microbial system. Basically on this knowledge a process monitoring and optimisation can be carried out for growth conditions.

To obtain reproducible experiments, a standard procedure for sample handling was developed by carrying out measurements in a batch calorimeter within a scale up to 20 ml on the bacterial culture *Paracoccus denitrificans*. The heat released during the growth process of the bacterial culture was detected and compared to the calculated value regarding the glucose within the growth medium as the sole energy-source. In additional experiments the oxygen supply within the calorimeter was varied in order to investigate its influence on the microbial activity. To get information about the usage of terminal electron acceptors available within the growth medium (oxygen, nitrate) the amount of nitrate was determined at the end of the calorimetric measurement by ion chromatography.

The methods used for these experiments are described in the following.

2. Materials, methods and instruments

2.1. Materials

The organism used for the measurements was *P. denitrificans* DSMZ strain 65. It is a gram-negative soil-bacterium with a strictly respiratory metabolism type. The cells are non-motile and aerobic. Anaerobic growth is possible if nitrate, nitrite or nitrous oxide are available as terminal electron acceptors [18]. Its ability to grow under aerobic and anaerobic condition allows measurements in the batch calorimeter without an air supply, which was one of the major reasons for its application.

The culture medium is a medium for chemolithotrophic growth recommended by the DSMZ (medium 81) for cultivation of *P. denitrificans*. It contains per 1000 ml, glucose 0.9 g as the energy- and carbon-source, KH_2PO_4 2.3 g, $Na_2HPO_4{\cdot}2H_2O$ 2.9 g, NH_4Cl 1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $NaHCO_3$ 0.5 g, $CaCl_2{\cdot}2H_2O$ 0.01 g, Fe(NH_4)citrate 0.05 g and trace elements. The culture medium contains dissolved oxygen and $Ca(NO_3)_2{\cdot}4H_2O$ 0.05 g per 1000 ml as terminal electron acceptors. The culture medium was sterilized under pressure at 120 °C for 2 h in an autoclave.

Materials for glucose analysis are 2,2′-azino-bis(3-ethylbenzotiazoline-6-sulfonic acid) diammonium salt (ABTS) from Fluka, *Aspergillus niger* glucose oxidase (GOD) and horseradish peroxidase (POD) from Serva.

2.2. Methods

2.2.1. Calorimetric experiments

All calorimetric experiments have been carried out in isoperibolic reaction calorimeters in batch-mode at 25 °C. The cell culture was grown on minimal medium with glucose until it reached the exponential growth phase. Then it was transferred to Eppendorf tubes and frozen at −25 °C. For the experiment a sample was thawed in a water bath at 25 °C. The sample was given into a glass ampoule. This ampoule was brought into the calorimetric vessel. The calorimetric vessel was filled with 20 ml culture medium. The calorimeter was brought to equilibrium temperature of 25 °C over night. After a stable baseline was established the measurement was started by breaking the ampoule. Analogous arrangements were chosen for all following measurements. During the measurements the culture was stirred with a stirrer speed depending on the calorimeter used (see below MBK-90: 200 rpm; LKB 8710: 600 rpm; TAM: 100 rpm). The headspace above the culture in the calorimetric vessel was about 20 ml.

2.2.2. Glucose analysis

For the glucose analysis a calorimetric measurement was started and stopped after reaching the growth pattern of interest. The culture broth was taken out of the calorimetric vessel and the cells were removed by centrifugation. The clear liquid was saved for the glucose analysis.

For the analysis the ABTS/GOD/POD-solution was prepared on demand and stored at 4 °C. The solution can be stored for 3 days. The liquid obtained from the culture broth was brought to appropriate dilutions. The ABTS/GOD/POD-solution was added and after an incubation time of 30 min the extinction of the coloured solution was measured at 405 nm [19].

2.2.3. Nitrate analysis

The samples for nitrate analysis were obtained as the samples for glucose analysis. After the cells had been removed from the culture broth, the nitrate content was analysed with the ion chromatograph.

2.3. Instruments

The calorimeters used were: the LKB precision calorimetry system type 8710, the metal block calorimeter MBK-90 [20] and the THERMOMETRIC-2277 thermal activity monitor (TAM). For detailed description see [21]. Measurements of the optical density of the cultures and glucose analysis

were carried out with the UV-Vis spectrometer UNICAM 8625. The nitrate analysis was performed on the DIONEX DX 100 ion-chromatograph with the column AS 9-HC.

3. Experimental results

3.1. Calorimetric experiments

Fig. 1 shows a typical power–time curve obtained by the calorimetric measurements of the microbial growth process with a small step after the break of the ampoule followed by a very short lag-phase and an exponential increase which passes into a stationary behaviour and finally a decrease of the curve down to the level of the baseline. The power–time curves have been obtained from the measured temperature-difference–time data by use of the TIAN-equation [22]. The experiments have been carried out in the 25 ml culture vessel of the MBK-90, with glucose as the sole carbon-source at 25 °C. Under the chosen conditions, the growth behaviour of *P. denitrificans* was detected reproducibly.

During the experiments with the cell culture the resources for the bacterial growth are:

glucose as carbon- and energy-source, $c_{(\text{Glucose})} = 5\,\text{mmol}\,\text{l}^{-1}$;
dissolved oxygen as electron acceptor, $c_{(O_2)} = 0.23\,\text{mmol}\,\text{l}^{-1}$;
nitrate as electron acceptor, $c_{(NO_3^-)} = 0.425\,\text{mmol}\,\text{l}^{-1}$.

The maximum thermal power released from the cell culture is $P_{(\text{max})} = (65 \pm 2)\,\mu\text{W}\,\text{ml}^{-1}$. The heat released during the growth process of *P. denitrificans* was calculated via integration of several power–time curves. For the cell culture under observation the average value of the heat released during the growth process is $q_{(\text{experiment})} = (-5.95 \pm 0.35)\,\text{J}\,\text{ml}^{-1}$ (Fig. 2).

The biomass formed with the resources provided by the culture medium was determined as cell dry mass. The cell dry mass obtained from the culture at the end of the calorimetric experiment was $(410 \pm 50)\,\mu\text{g}\,\text{ml}^{-1}$. Accordingly, about 50% of the given energy-source (glucose) are transformed into biomass. The remaining 50% are released as heat. This is in good agreement with the literature [23,24].

Furthermore, the formula for the produced biomass was determined by elemental analysis. The formula obtained for *P. denitrificans* is $CH_{1.76}N_{0.21}O_{0.52}$. This formula corresponds to the literature [24,7] $CH_2N_{0.23}O_{0.52}$ with a formula weight of $25.54\,\text{g}\,\text{mol}^{-1}$. This formula is used at the further calculations. With the specific enthalpy of combustion from literature [25] $\Delta_c H = (-23.1 \pm 0.5)\,\text{kJ}\,\text{g}^{-1}$ and the formula weight, the molar enthalpy of combustion for the biomass was calculated: $\Delta_c H_{\text{Bio}} = (-591 \pm 13)\,\text{kJ}\,\text{mol}^{-1}$. This value allows the calculation of the molar ethalpny of formation of the biomass $\Delta_f H_{\text{Bio}}$. The calculation is based on the following reaction equation:

$$CH_2N_{0.23}O_{0.52} + 1.24O_2 \rightarrow CO_2 + H_2O + 0.115N_2 \quad (1)$$

The corresponding standard enthalpies are given in literature [26].

With $\Delta_f H_{\text{Bio}} = \Delta_f H_{CO_2} + \Delta_f H_{H_2O} - \Delta_c H_{\text{Bio}}$ a molar enthalpy of formation is obtained of $\Delta_f H_{\text{Bio}} = -88.1\,\text{kJ}\,\text{mol}^{-1}$.

According to the given resources the glucose oxidation is possible by the following reaction paths:

- oxidation with dissolved oxygen;

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O,$$
$$\Delta_R H = -2935.6\,\text{kJ}\,\text{mol}^{-1} \quad (2)$$

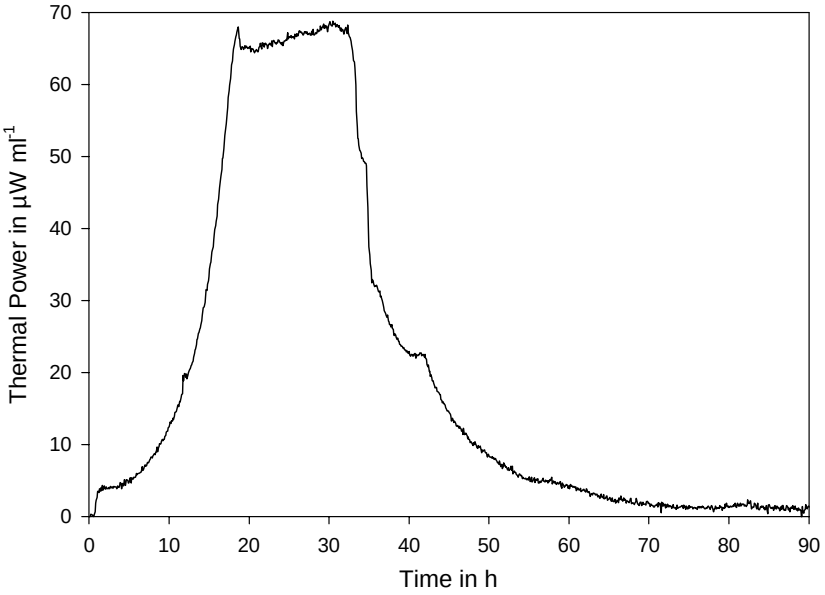

Fig. 1. Power–time curve of the growth process of *Paracoccus denitrificans* in glucose medium at 25 °C.

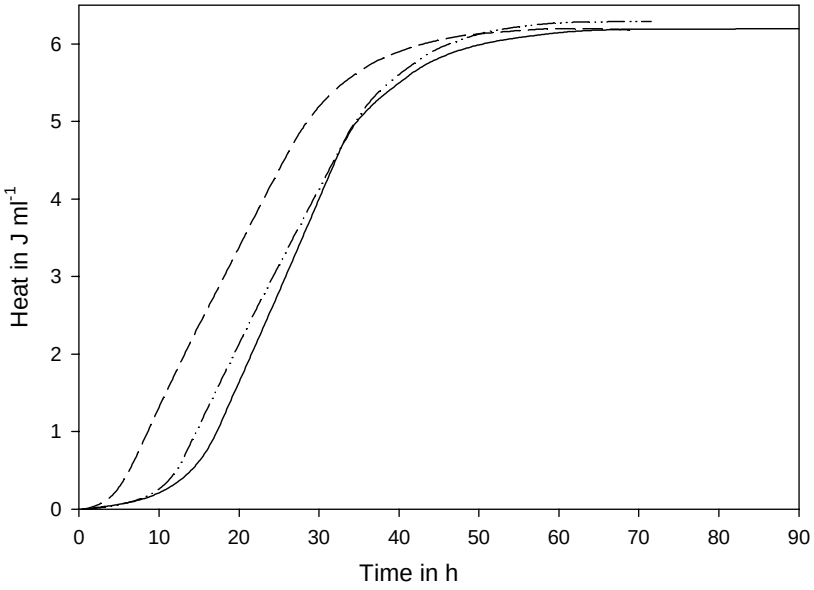

Fig. 2. Calculated heat–time curves of the growth process of *Paracoccus denitrificans* in glucose medium at 25 °C.

- oxidation with nitrate;

$$C_6H_{12}O_6 + 4.8NO_3^- \rightarrow 6CO_2 + 3.6H_2O + 4.8OH^- + 2.4N_2,$$
$$\Delta_R H = -2386.1\,\mathrm{kJ\,mol^{-1}} \quad (3)$$

Corresponding to Eqs. (2) and (3) a heat of $q_{(\mathrm{calculated})} = -0.324\,\mathrm{J\,ml^{-1}}$ is expected during the experiment. This is only 5.4% of the heat detected in the experiments of $q_{(\mathrm{experiment})} = (-5.95 \pm 0.35)\,\mathrm{J\,ml^{-1}}$. To explain the high heat value from the experiment not only the dissolved electron acceptor has to be regarded. During the growth process additional oxygen can diffuse into the culture broth from the gas phase.

The glucose analysis showed that the glucose is completely converted during the growth process (see below). With a nitrate concentration of $0.425\,\mathrm{mmol\,l^{-1}}$, according to Eq. (3), a heat of $q = -0.21\,\mathrm{J\,ml^{-1}}$ can be obtained. This heat is very low compared to the heat obtained from the complete glucose oxidation with oxygen (Eq. (2)) of $q = -14.68\,\mathrm{J\,ml^{-1}}$. Therefore the nitrate is neglected. Furthermore, the production of biomass during the bacterial growth process must be regarded. With this information a more convenient equation for the growth process can be formulated.

$$C_6H_{12}O_6 + 0.69NH_3 + 2.7975O_2 \rightarrow 3CO_2 + 4.035H_2O + 3CH_2N_{0.23}O_{0.52},$$
$$\Delta_R H = -1309\,\mathrm{kJ\,mol^{-1}} \quad (4)$$

The nitrogen source is available as ammonium ions in the culture medium and enters the reaction equation as ammonia. For Eq. (4) the enthalpy of reaction is calculated from the enthalpies of formation. Regarding Eq. (4) the expected heat during the cultivation is $q_{(\mathrm{calculated})} \approx -6.5\,\mathrm{J\,ml^{-1}}$. This result corresponds very well with the experimental data $q_{(\mathrm{experiment})} = (-5.95 \pm 0.35)\,\mathrm{J\,ml^{-1}}$. Eq. (4) is therefore suitable for the characterisation of the investigated microbial process.

3.2. Glucose analysis

For observation of the glucose consumption during the growth process, several calorimetric experiments have been stopped after showing the growth pattern of interest. The culture broth was centrifuged and the remaining liquid part used for glucose analysis.

Fig. 3 shows the conversion of glucose and nitrate together with the heat detected during the growth process. The main consumption of the glucose occurs during the exponential growth of the cell culture. About 40 h after the break, the glucose concentration decreases below the concentration, which can be detected with the applied test ($<1\,\mu\mathrm{g\,ml^{-1}}$). The effect of the glucose deficiency becomes apparent from the heat–time curve as well as from the power–time curve, whereas the curves show a different behaviour. Under glucose deficiency, the heat–time curve becomes stationary (Fig. 3) similar to the behaviour of a common growth curve obtained by photometric methods. In contrast to the heat–time curve, the power–time curve shows a steep decrease under glucose shortage ($t > 35$ h, see Fig. 1). Obviously the power–time curve gives direct information about the activity of the cell culture. Because of the different information obtained from the heat–time curve and the power–time curve a different terminology is used for their discussion. In particular the “stationary part” of the power–time curve is a stationary part from the energetic point of view in contrast to the stationary phase defined for microbial systems. Therefore it is mentioned as stationary part in the following discussion.

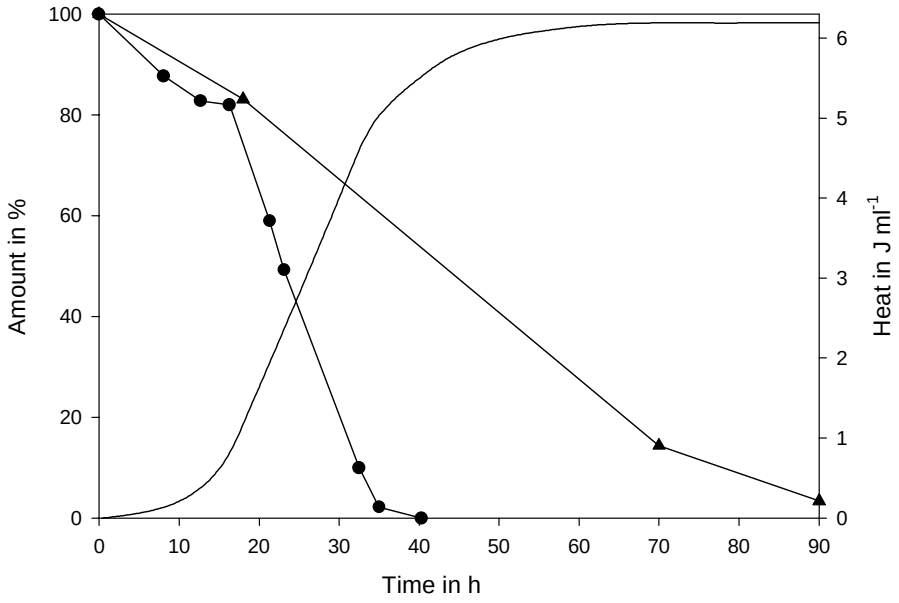

Fig. 3. Calculated heat–time curve in correlation with the consumption of glucose and nitrate during the cultivation: (—) heat–time curve; (●) concentration of glucose; and (▲) concentration of nitrate.

3.3. Variation of the oxygen supply

In order to get information about the influence of the oxygen supply on the microbial activity, several measurements were carried out in the LKB calorimeter under variation of the oxygen supply. The calorimetric vessel was filled with 80 ml of culture medium, and the mass of cell suspension used as inoculum was 700 mg. Under these conditions several measurements were carried out without additional gas supply as a reference for the experiments under variation of the oxygen supply. For a better supply of the cell culture with electron acceptor, oxygen was directly brought into the culture broth at a flow rate of 24 ml h^{-1}. For anaerobic conditions in the culture broth, argon was brought into it at the same flow rate. Fig. 4 shows several curves from these experiments illustrating the changes in microbial activity. Without additional gas supply a thermal power of $P_{(\max)} = (111 \pm 13)\,\mu\mathrm{W\,ml^{-1}}$ was detected. Furthermore, these experiments show a stationary part at the end of exponential part of the power–time curve. With oxygen supply a three times higher thermal power compared to the experiments without gas supply of $P_{(\max)} = (343.5 \pm 27.6)\,\mu\mathrm{W\,ml^{-1}}$

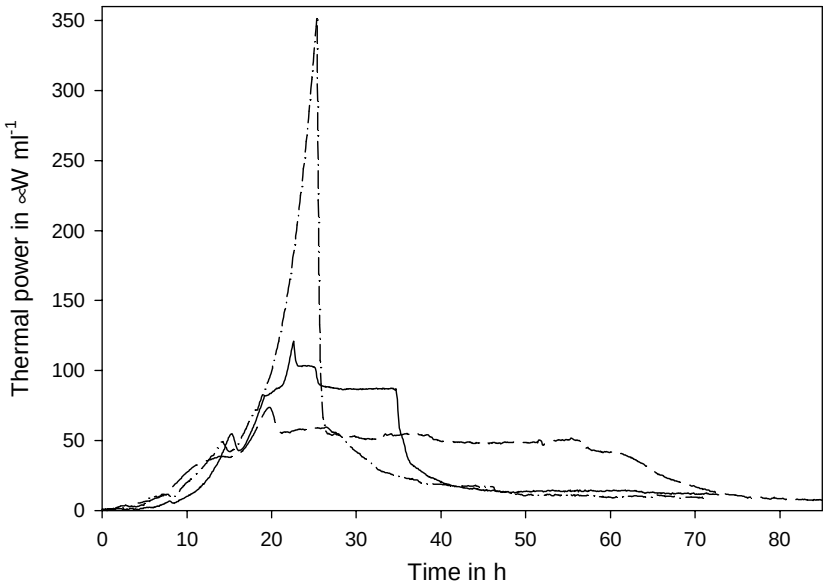

Fig. 4. Calorimetric experiments under variation of oxygen supply: (—) experiment without additional gas supply; (—·—) experiment under oxygen supply; and (– – –) experiment under argon atmosphere.

was detected. The measurements under oxygen supply show a sharp decrease at the point were the given carbon source (glucose) is consumed. Under oxygen supply no stationary part was observed. During the experiments under argon atmosphere it was not possible to exclude oxygen completely but the oxygen content in the growth medium was reduced considerably. It can be seen, that the thermal power becomes significantly smaller if the oxygen supply is reduced. The thermal power detected under argon atmosphere was $P_{(max)} = (68.0 \pm 9.9)\ \mu W\,ml^{-1}$. The experiments under argon atmosphere show the expected behaviour. Because of the low oxygen content, the stationary part appears earlier and lasts longer than in the experiments with better oxygen supply. These experiments show that the occurrence of the stationary part is an effect of the oxygen limitation. The level of the stationary part is determined by the oxygen concentration available in the culture broth. The stationary part finishes when the glucose available is converted. Therefore the length of the stationary part depends on the oxygen concentration and the glucose concentration available in the growth medium.

Within the experimental uncertainties, the areas under the curves in Fig. 4 are equal.

3.4. Nitrate analysis

Besides oxygen as terminal electron acceptor for aerobic growth, nitrate is available as terminal electron acceptor for anaerobic growth. For information about the use of nitrate during the growth process, several calorimetric measurements, carried out with the MBK-90 without additional gas supply, have been stopped at the beginning of the exponential part and at the end of the calorimetric experiment. The cells were removed from the culture broth and the clear liquid obtained was analysed with an ion chromatograph.

Fig. 3 shows the amount of nitrate remaining in the culture broth during the cultivation. The culture medium contains $26.4\ \mu g\,ml^{-1}$ (according to 3.1) at the beginning of the experiment. After 18 h of cultivation about 80% of the initial nitrate concentration ($(21.95 \pm 0.21)\ \mu g\,ml^{-1}$) were detected. At the end of the cultivation about 14% of the initial nitrate concentration ($(3.8 \pm 1.0)\ \mu g\,ml^{-1}$) were still available. The results of further measurement after 90 h were smaller than the detection limit of $1\ \mu g\,ml^{-1}$.

It was expected that the nitrate as electron acceptor for anaerobic growth is used when oxygen as electron acceptor for aerobic growth becomes limiting. When the oxygen content of the culture broth is too low to ensure the further exponential growth of the cell culture, the stationary part occurs in the calorimetric curve. It is most likely that the major amount of nitrate is used as electron acceptor at the end of the exponential part of the thermal power curve. Since only a few measurements of the nitrate concentration were carried out, the data only shows the tendency of the nitrate consumption during the growth process. On the basis of these results further investigations on the nitrate consumption seem to be necessary.

4. Discussion

The power–time and the heat–time curves obtained from the calorimetric measurements give different information about the bacterial growth process. *The power–time curves include the information about the current microbial activity*. Changes in the slope of the power–time curves show the effects of limiting factors on the microbial activity during the cultivation. In Fig. 1 a two-fold limitation is apparent. The first limiting factor is the available oxygen. Its influence on the activity of the bacterial culture becomes visible about 16 h after the start of the cultivation by the transition from the exponential phase of the power–time curve to the stationary part. Due to the continuous diffusion of oxygen from the gas phase of the calorimetric vessel into the culture broth the stationary part lasts for 20 h. After the stationary part the thermal power detected from the cell culture decreases drastically. This behaviour is the effect of the glucose limitation as the second limiting factor. From the decreasing phase the power–time curve does not directly return to the baseline but to a level slightly above the baseline. This shows, that the cells are still active for a long period after the glucose is completely converted. On the basis of the power–time curve, the behaviour of the cell culture can be discussed as follows.

At the beginning of the experiment a small volume of cell suspension (700 μl) is brought into a much larger volume of culture medium (20 ml). Therefore the cell number in the culture broth is very low and there are no limitations. After the lag-phase the cells are adapted to the culture medium and the cell culture starts the exponential growth. During the exponential growth phase, the thermal power and the heat exchanged from the cell culture show an exponential increase as well. With the increase in cell number and the conversion of glucose with oxygen during the exponential growth, the oxygen demand of the cell culture rises and finally exceeds the amount of oxygen available in the culture broth. At this point oxygen becomes limiting and terminates the increase in cell number. Further growth of the cell culture is not possible any longer. Owing to the constant diffusion of oxygen from the headspace of the calorimetric vessel into the culture broth, the available cells are still able to respire glucose. Under these conditions the diffusion rate of oxygen controls the glucose consumption, indicated by the begining of the stationary part. It can be assumed, that the oxygen concentration during the stationary part of the power–time curve is nearly zero because the oxygen diffusing into the culture broth is utilised immediately. In order to validate these assumptions, experiments using an oxygen sensor are planned.

In contrast to the power–time curves, which show the current activity of the cell culture, the heat–time curves

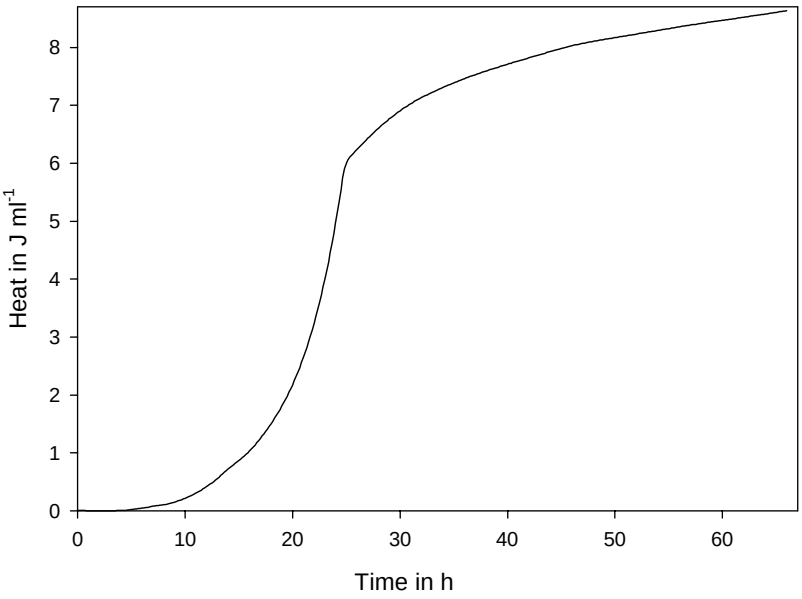

Fig. 5. Calculated heat–time curve of the experiment under oxygen supply.

show the entire heat exchanged by the cell culture with the environment during the cultivation. Fig. 3 shows the heat–time curve together with the glucose and the nitrate conversion. Compared to the glucose conversion, the heat–time curve shows a similar behaviour to the growth curves usually obtained in microbiology by measurements of the optical density at a certain wavelength. Regarding the similarities between the growth curves and the heat–time curves, the pattern appearing in the heat–time curve can be discussed as an analogue to the pattern of a growth curve.

The heat–time curve (Fig. 3) as well as the power–time curve (Fig. 1) show an exponential increase until the first limitation (by oxygen in the given example) occurs (about 16 h after the start of the cultivation). Due to the change of the power–time curve to a stationary behaviour, the heat–time curve, as the integral of the power–time curve, changes to a linear increase.

In the experiments under additional supply of oxygen, glucose is the sole limiting factor. For comparison the heat–time curve of an experiment under oxygen supply (Fig. 4) is given in Fig. 5. It shows a distinct exponential increase until the growth is limited by the glucose after 24 h. With the complete conversion of the glucose the cell culture lacks its carbon-source for production of new biomass. Under these circumstances the cells can utilise storage substances and their own cell material for endogenous metabolism. This enables the cells to stay active over a long period of time. Therefore measurements of the optical density of a cell culture under these conditions show a stationary behaviour. The same information can be obtained from the heat–time curve. In contrast to the heat–time curve, the power–time curve shows a steep decrease when the glucose limitation becomes relevant. The endogenous metabolism lies at a significant lower level of thermal power than the glucose conversion under oxygen limitation.

5. Conclusions

The experiments with *P. denitrificans* show that it is possible to follow the microbial growth behaviour by calorimetric means and that the results obtained are reproducible if a standardised treatment of the pre-cultures is applied and the pre-cultures are stored as frozen samples.

The calorimetry provides information about the activity and growth behaviour of the cell culture, which is very important for controlling of production processes in biotechnology.

In addition the calorimetry brings its inherent advantages of being non-specific, non-invasive and insensitive to the electrochemical and optical properties of the system under observation. This means calorimetry is applicable even for an inhomogeneous culture broth or cultivations under formation of turbid products where photometric methods cannot be applied.

With the experiments performed and the value of heat exchanged during the growth process it was possible to find a reaction Eq. (4) for the metabolism of the cell culture under observation. The heat calculated from this equation corresponds very well to the experimental data. Thus the obtained reaction equation is a suitable model for the metabolism of the examined cell culture.

In order to correlate the calorimetric curves to the bacterial growth behaviour, additional analyses on terminal electron acceptors and carbon source have been carried out. The limitation by electron acceptors and carbon source are leading to significant changes in the

growth behaviour, which can be followed clearly by calorimetry.

For further work it is planned to correlate the calorimetric curve with the photometric experiment. Therefore the measurement of the optical density and other parameters (oxygen-, CO_2-content) will be carried out simultaneous with the calorimetric experiment in the calorimetric vessel. When this correlation is properly found for the standard organism (*P. denitrificans*), the calorimetric method can be applied at cell cultures where the investigation by photometric means is not possible.

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