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Calorimetric investigations on the degradation of water insoluble hydrocarbons by the bacterium *Rhodococcus opacus* 1CP

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ABSTRACT

The growth of the bacterium *Rhodococcus opacus* 1CP on water insoluble carbon sources (*n*-tetradecane and *n*-hexadecane) has been investigated by calorimetry, using the LKB 8700 device. The applied method proved to be well suited for process-monitoring in heterogeneous systems. High reproducibility was obtained for cultivation on the chosen hydrocarbons. Equations for the growth process were constructed based on yield coefficients for the products of the microbial growth. Good correspondence of experimental and calculated enthalpy change was accomplished by regarding the production of biosurfactants as additional product.

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1. Introduction

Intensive use of petroleum products is strongly connected to the anthropogeneous discharge of hydrocarbons into the environment. Hence hydrocarbons are widespread pollutants, the hydrophobicity of which causes a low bioavailability and therefore a particular persistence against bioremediation measures. However, numerous terrestrial and aquatic species of microorganisms possess the ability to degrade water insoluble hydrocarbons. In order to increase the bioavailability of the otherwise hardly accessible substrates, microorganisms use strategies like increasing the hydrophobicity of their surfaces or the excretion of emulsifiers [1].

Among others, members of the genus *Rhodococcus* have the ability to produce biosurfactants during their growth on long-chain hydrocarbons [2]. The majority of the biosurfactants formed by *Rhodococcus* strains are classified as glycolipids [3] which are the most important and yet best investigated biosurfactant class [4]. Glycolipids show a high surface activity, high pH and temperature stability. Compared to their synthetic counterparts, glycolipids are considered to be readily biodegradable with lower toxicity [5]. The antimicrobial activity [6] and good technical characteristics [7] discussed for glycolipids open fields of application for these biosurfactants ranging from bioremediation, food and agrochemical industry to pharmaceutics. The investigation of the microbial alkane degradation and the synthesis of biosurfactants is, therefore, from an ecological point of view, of particular interest.

However, the growth behaviour of these systems cannot be investigated by classical microbiological methods (photometric methods or methods which require defined sampling like protein estimation, dry mass or cell number determination), due to the aggregation of cells leading to heterogeneous cell cultures. In contrast the application of calorimetric methods allows a contemporary, continuous and non-invasive monitoring of the microbial activity even for such heterogeneous systems. Furthermore calorimetry provides the opportunity to investigate wide cell-density ranges and the particular noticeable microbial growth strategies.

For the investigation of microbial alkane degradation the metabolic versatile strain *Rhodococcus opacus* 1CP was chosen as model organism. According to the investigations given in [1] *R. opacus* 1CP is able to utilise long chained *n*-alkanes (C_{10}–C_{16}) as sole energy- and carbon-source. By thin-layer-chromatographic analysis of organic extracts of these cultivations a trehalose lipid (glycolipid) was detected and identified as trehalose dinocardiomycolate. Concerning the cultivation of trehaloselipid producing strains high product yields are discussed for the use of *n*-tetradecane, *n*-pentadecane or *n*-hexadecane [8]. Therefore the cultivation of *R. opacus* 1CP in the calorimeter was carried out using either *n*-tetradecane or *n*-hexadecane as sole energy and carbon source.

The cultivation of *R. opacus* 1CP on *n*-alkanes was recorded calorimetrically. For exact consideration of the products,

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elementary composition and yield coefficients of biomass and biosurfactant were determined. Based on these experimental data reaction equations were drawn up, the enthalpy of reaction was calculated and verified by the experimental results from the calorimetric measurements.

2. Experimental

2.1. Microorganism Rhodococcus opacus 1CP

The bacterial strain *R. opacus* 1CP was first isolated from soil in enrichment culture on 2,4-dichlorophenol [9]. Metabolic pathways for chloroaromatic compounds (mono-, di-, and tri-chlorophenols) are discussed and illustrate its versatility [10]. Furthermore it has the ability to utilise benzoate, 4-hydroxybenzoate and 4-chlorophenol [9] as well as long chained *n*-alkanes (C_{10}–C_{16}) as sole energy and carbon source [1].

2.2. Culture medium

The culture was grown on agar plates with a mineral medium [11] containing 2 mmol l^{-1} benzoate as energy and carbon source. The cultivation temperature was 30 °C and the storage temperature 4 °C. A standard- culture medium for chemolithotrophic growth was used for providing defined conditions during the calorimetric experiments. The culture medium consists of a mineral solution with additional 0.1 g l^{-1} $(NH_4)_2SO_4$ as a further nitrogen source. It contains per 1000 ml 2 g KH_2PO_4, 7 g $Na_2HPO_4{\cdot}2H_2O$, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.01 g ferric ammonium citrate, 1 g $(NH_4)_2SO_4$, 0.05 g $Ca(NO_3)_2{\cdot}4H_2O$ and the trace element solution SL 6 [12].

2.3. Calorimetric experiments

The inoculum for the calorimetric experiments was prepared in liquid pre-cultures grown in baffled Erlenmeyer flasks (culture volume 200 ml) at 30 °C and agitation with 132 rpm on the same medium and carbon source used for the experiment. After an incubation time of 12 days the carbon source was exhausted. The cells were harvested and washed five times with sterile sodium chloride solution. The inoculum samples contained a cell dry mass of 19 mg.

The measurements were done in two reaction calorimeters (both type LKB 8700) at 25 °C. Detailed description of the calorimeter is given in [13] and its application for the investigation of microbial growth is illustrated in [14]. The 100 ml calorimetric vessels were made of glass equipped with stirrers (stirring speed 200 rpm). In order to meet the demands of aerobic cultivations on hydrocarbons the vessels were additional equipped with an oxygen supply system and a titration capillary, connected to a syringe pump, for the addition of the water insoluble carbon source. The oxygen supply of the culture was studied in preliminary experiments with an amperometric oxygen sensor [15]. During the experiment the supply system provides an oxygen concentration of 0.89 mmol O_2 l^{-1} which corresponds to 73% oxygen saturated water [16]. Heat effects from evaporation were avoided by saturation of the oxygen with water vapour. All calorimetric experiments were carried out in liquid culture using the mineral medium described above. The inoculum sample (1.5 ml) together with 90 ml culture medium without any carbon source was placed in the sterilised calorimetric vessel. The oxygen supply system and the titration capillary were connected to the calorimetric vessel and it was inserted into the thermostatic bath. The calorimeter was brought to equilibrium temperature of 25 °C. At this temperature the calorimetric system was tested during a time period comparable to the experimental runs. Changes of the baseline over a long period of time (48 h) are lower then ±0.5 mK. In the same period no detectable systematic rise of the baseline is observable. After stabilisation of the baseline the experiment was started by titration of 50 μl of the appropriate alkane into the culture.

As the result of the calorimetric measurement using the LKB 8700 a time-dependent temperature difference $\Delta T(t)$ was obtained. From the ΔT-t-data thermal power–time data $P(t)$ were calculated using the TIAN equation [17]:

$$P(t) = \frac{\Delta T(t)}{R_{th}} + c_p \times \frac{d\Delta T(t)}{dt} \quad (1)$$

As preconditions for these calculations reliable and constant values for R_{th} and c_p have to be determined, especially regarding long time measurements. A convenient method for determining R_{th} was presented in earlier publications. This calibration method, heating with a constant power input up to thermal equilibrium, is illustrated for the determination of the enthalpy of crystallisation of lithium chloride by measurements of the partial molar dissolution enthalpies near saturation [18] and was applied for our system. By electrical calibration after each experiment this parameter was validated and the c_p was determined. The calculation of the enthalpy change was done by integration of the thermal power–time curve.

The verification of our results was possible using a further calorimetric system. Comparative measurements were carried out with the TAM II, which are a part of a further publication. The accordance of the ΔH values confirms the usability of our calorimetric system for long time investigations.

2.4. Biomass analysis

According to the pre-cultures for the calorimetric measurements *R. opacus* 1CP was grown in liquid culture on the appropriate *n*-alkane. After the carbon source was exhausted the cells were harvested. For the determination of the elemental composition of the biomass the culture medium was removed by washing the cells with sterile tap water, the remaining liquid was removed by centrifugation and the biomass was dried at 110 °C for 10 h. Elemental analyses were done by the elementary analytical laboratory using a CHN-O-RAPID device from the firm Heraeus. The determination of the biomass yield coefficient was done by dry mass determination according to the membrane filtration method [19].

2.5. Biosurfactant quantification

For the determination of the biosurfactant yield coefficient the anthron-sulfuric acid method was used, which is well established for the quantification of sugar contents [20]. The method is based on the oxidation of the sugar moiety (trehalose) of the biosurfactant molecule (trehalose dinocardiomycolate). The biosurfactant quantification was done with the obtained culture broth from cultivation of *R. opacus* 1CP in the calorimeter. After the calorimetric experiment was finished the calorimetric vessel was taken out of the thermostatic bath and the culture broth was saved for further analysis. For extraction of the biosurfactant the biomass was separated from the culture medium by filtration (frit pore width 10–16 μm). The biomass was washed three times with 10 ml chloroform and the organic phases were collected. After vaporisation of the chloroform the biosurfactant crude extract was obtained. By hydrolysis with 1 N sodium hydroxide solution at 90 °C for 1 h the ester bonds of the biosurfactant molecule were destroyed. Then the reaction batch was neutralised with hydrochloric acid solution and the solid particles were removed by centrifugation. The clear supernatant containing the trehalose residue was analysed using the anthron-sulfuric acid method by photometric measurement at 620 nm. The quantification of the trehalose content was done by reference to a calibration curve.

3. Results and discussion

3.1. Power–time data

The power–time curves display the current metabolic activity of the cells and hence contain information about the influence of cultivation conditions to the culture. Changes in growth behaviour due to limitations or occurrence of toxic intermediates can clearly be registered in the power–time curve [21].

Interpretation of the various cultivations demands a system referring thermal power. Therefore the measurements were carried out under equal experimental conditions and at constant volumes for culture medium and inoculum. The amount of substance of the carbon source was 170 μmol for cultivation on *n*-hexadecane and 192 μmol for cultivation on *n*-tetradecane. Fig. 1 shows power–time curves obtained from five cultivations of *R. opacus* 1CP on *n*-hexadecane. The growth behaviour results from the interaction of the bacterial strain with the water insoluble carbon source and is characteristic for these cultivations. At the start of the experiment the bacterial cells have access to all required substrates (i.e. water, carbon source, oxygen, nitrogen source). Hence, the growth process appears unlimited for about 20 h. The distinct change in the slope of the power–time curves above 20 h (Fig. 1) displays the occurrence of a limitation. The development of cell-aggregates (laser diffraction, results not shown) among the hydrophobic carbon source causes a limitation due to reduced access of the bacterial cells to the substrates dissolved in the aqueous culture medium. Because of the high sensitivity of the cells degrading alkanes against oxygen limitation the access to oxygen appears to play the major role for the decline in thermal power. About 33 h after start of the experiment the curves decrease gradually. During this period the alkane is still utilised but the number of cells provided with all necessary substrate decreases steadily. About 95 h after start of the experiment the power–time curves (Fig. 1) appear nearly parallel to the baseline but show an offset. At this time the carbon source is depleted whereas cells are still active and contribute a thermal power by their maintenance metabolism.

Regarding the heterogeneous system the power–time curves show good reproducibility. Recording of power–time data therefore appears as a suitable instrument for process-monitoring of microbial cultivations. Together with additional analytics (i.e. concerning carbon source content and aggregate development) calorimetric measurements permit the discussion of the cell–substrate interaction during the cultivation.

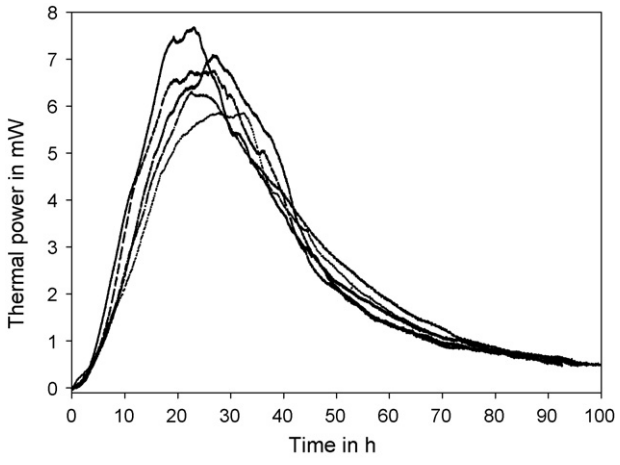

Fig. 1. Power–time curves of the growth process of *R. opacus* 1CP on 192 μmol *n*-hexadecane at 25 °C.

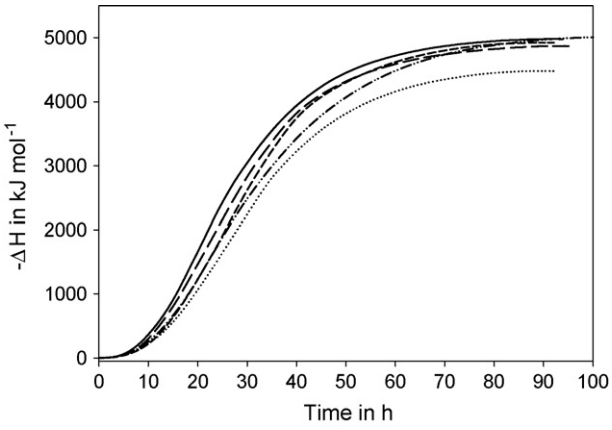

Fig. 2. Enthalpy–time curve of the growth process of *R. opacus* 1CP on *n*-hexadecane at 25 °C.

3.2. Enthalpy–time data

By integration of the area under the power–time curve the heat exchanged during the cultivation is obtained. Because the microbial growth process runs at constant pressure the exchanged heat can be discussed as an enthalpy change. For indication of an intensive quantity the exchanged heat was referred to the amount of substrate. Fig. 2 shows the enthalpy–time curves for the cultivation of *R. opacus* 1CP on *n*-hexadecane. The enthalpy–time curves, as an integral signal, can be discussed in accordance to a common growth curve obtained from photometric or respirometric measurements which are already well established for microbiological investigations.

The curves indicate the good reproducibility of the cultivation under the chosen culture conditions. When the carbon source is consumed, the exchanged heat reaches its maximum shown as plateau in the enthalpy–time curve. The plateau value of the enthalpy–time curve is of particular interest because it displays the total heat exchanged during the microbial growth process and hence the enthalpy of the process. This value is also a suitable measure for the reproducibility of the cultivation. As shown in Fig. 2 for the growth process of *R. opacus* 1CP on *n*-hexadecane an enthalpy of $\Delta_R H = (-4690 \pm 80)\,\mathrm{kJ\,mol^{-1}}$ was determined from the calorimetric measurements. In the following the experimentally determined enthalpy value is used for verification of reaction equations for the growth process drawn up based on yield coefficients.

3.3. Reaction equations from experimental yield coefficients

Although microbial growth is combined with the proceeding of a wide variety of chemical reactions, from a thermodynamical point of view even these complex processes can be summarised in one single reaction equation [22–25].

The aerobic microbial growth on a carbon source with the formula (C_xH_y) can be regarded according to the equation:

$$C_xH_y + aO_2 + bNH_3 \rightarrow cCH_rO_sN_t + dCO_2 + eH_2O \quad (2)$$

In this equation the product species ($CH_rO_sN_t$) is the biomass formed during the microbial growth. Based on Eq. (2) an enthalpy of reaction can be calculated for the microbial growth process. This requires knowledge of the enthalpies of formation of all involved species. Usually these values are well documented. However, for biomass and complex biomolecules formation enthalpies are not yet available. Hence the enthalpy of formation for biomolecules has to be calculated from the enthalpy of combustion. A well established approach is the method of estimating the stan-

dard enthalpy of combustion from the reductance degree of a biomolecule as discussed by Cordier et al. [26]. The application of this method requires the knowledge of the elemental composition of the regarded biomolecules. For the biomass of *R. opacus* 1CP growing on *n*-alkanes the composition was determined by elemental analysis of dry biomass samples. The elemental composition of the alkane-grown biomass corresponds to the formula $CH_{1.85}O_{0.37}N_{0.11}$. Regarding the oxycaloric quotient according to Giese [27] of $Q = 115.06\,\mathrm{kJ\,(e^-\,mol)^{-1}}$ the standard enthalpy of combustion for the alkane-grown biomass of *R. opacus* 1CP is $\Delta_c H^\circ_{\mathrm{Biomass}} = -588.0\,\mathrm{kJ\,C\text{-}mol^{-1}}$. According to Eq. (3) the standard enthalpy of formation can be calculated from the standard enthalpy of combustion:

$$\Delta_f H^\circ_{\mathrm{Biomass}} = \Delta_f H^\circ_{CO_2} + \Delta_f H^\circ_{H_2O} - \Delta_c H^\circ_{\mathrm{Biomass}} \quad (3)$$

The value of $\Delta_f H^\circ_{\mathrm{Biomass}} = -69.9\,\mathrm{kJ\,C\text{-}mol^{-1}}$ was calculated for the alkane-grown biomass of *R. opacus* 1CP.

Stoichiometric coefficients of the involved species are needed in order to draw up reaction equations. Assuming the carbon source is transformed during the microbial growth process to biomass and CO_2, the yield coefficient of one product species is sufficient for drawing up the equation. The biomass yield coefficient can be easily obtained from dry mass determination. In the present case the determination was done by the membrane filtration method. The biomass yield coefficients for cultivation of *R. opacus* 1CP on the alkanes *n*-tetradecane and *n*-hexadecane are:

Cultivation on *n*-tetradecane :

$$Y_{X/S} = 0.98 \pm 0.06\,\mathrm{g\,biomass/g}\,n\text{-tetradecane}$$

Cultivation on *n*-hexadecane :

$$Y_{X/S} = 0.97 \pm 0.05\,\mathrm{g\,biomass/g}\,n\text{-hexadecane}$$

This data corresponds to an amount of substance of 9.1 C-mol biomass formed when growing on 1 mol *n*-tetradecane and 10.3 C-mol biomass formed when growing on 1 mol *n*-hexadecane. For the cultivation on *n*-tetradecane the resulting reaction equation reads:

$$C_{14}H_{30} + 10.63O_2 + 1NH_3 \rightarrow 9.1CH_{1.85}O_{0.37}N_{0.11} + 4.9CO_2 + 8.09H_2O \quad (4)$$

Using the database [28] the enthalpy of reaction for Eq. (4) was calculated as $\Delta_R H_{(\mathrm{calculated})} = -4366\,\mathrm{kJ\,mol^{-1}}$.

If the reaction equation is a suitable model for the investigated growth process, the calculated enthalpy value has to correspond to the experimentally determined enthalpy from calorimetric data. In case of the cultivation of *R. opacus* 1CP grown on *n*-tetradecane the calorimetrically determined enthalpy is $\Delta_R H_{(\mathrm{experimental})} = (-3770 \pm 16)\,\mathrm{kJ\,mol^{-1}}$. Compared to the calculated value a significant difference occurs and hence the given Eq. (3) is not convenient for the investigated growth process. However, the calculated value, being significantly more negative than the experimental value, indicates at least one additional product neglected in the reaction equation. As mentioned above, when growing on hydrocarbons *R. opacus* 1CP is able to form biosurfactants. The biosurfactant formed by *R. opacus* 1CP is a trehalose dinocardiomycolate with the elemental composition $CH_{1.88}O_{0.13}$ referred to the amount of carbon. Detailed description of the biosurfactant structure is given by Schlömann and co-workers [1]. The knowledge of the elemental composition of the biosurfactant permits the calculation of the enthalpy of formation as done for the biomass. Using the method described above an enthalpy of formation of $\Delta_f H^\circ_{\mathrm{Biosurfactant}} = -15.6\,\mathrm{kJ\,C\text{-}mol^{-1}}$ was calculated.

After the microbial growth process was finished, several biosurfactant quantifications were done with culture broths, obtained from calorimetric experiments. Even when the carbon source was consumed and no further microbial activity was detected the biosurfactant was present in the culture broth at maximum yield. For the cultivations of *R. opacus* 1CP, carried out on the carbon sources *n*-tetradecane and *n*-hexadecane, the following yield coefficients were obtained from quantification using the anthron-sulfuric acid method:

Cultivation of *Rhodococcus opacus* 1CP on *n*-tetradecane :

$$Y_{P/S} = (0.079 \pm 0.005)\,\mathrm{g\,biosurfactant/g}\,n\text{-tetradecane}$$

Cultivation of *Rhodococcus opacus* 1CP on *n*-hexadecane :

$$Y_{P/S} = (0.040 \pm 0.002)\,\mathrm{g\,biosurfactant/g}\,n\text{-hexadecane}$$

The biosurfactant quantification after the calorimetric experiment indicates, that the biosurfactant is not metabolised after the carbon source is consumed. Therefore the biosurfactant has to be regarded as a product of the microbial growth process. The stoichiometric coefficients for regarding the biosurfactant in the reaction equation can be calculated from the yield coefficients. For cultivation of *R. opacus* 1CP 0.98 mol biosurfactant were obtained when growing on 1 mol *n*-tetradecane and 0.57 mol biosurfactant when growing on 1 mol *n*-hexadecane. Regarding these data the reaction equations for the investigated growth process were modified as follows:

Cultivation of *R. opacus* 1CP on *n*-tetradecane:

$$C_{14}H_{30} + 9.25O_2 + 1NH_3 \rightarrow 9.1CH_{1.85}O_{0.37}N_{0.11} + 3.92CO_2 + 7.16H_2O + 0.98CH_{1.88}O_{0.13} \quad (5)$$

$\Delta_R H_{(\mathrm{calculated})} = -3729\,\mathrm{kJ\,mol^{-1}}$; $\Delta_R H_{(\mathrm{experimental})} = (-3770 \pm 16)\,\mathrm{kJ\,mol^{-1}}$

Cultivation of *R. opacus* 1CP on *n*-hexadecane:

$$C_{16}H_{34} + 11.39O_2 + 1.13NH_3 \rightarrow 10.3CH_{1.85}O_{0.37}N_{0.11} + 5.13CO_2 + 8.64H_2O + 0.57CH_{1.88}O_{0.13} \quad (6)$$

$\Delta_R H_{(\mathrm{calculated})} = -4641\,\mathrm{kJ\,mol^{-1}}$; $\Delta_R H_{(\mathrm{experimental})} = (-4690 \pm 80)\,\mathrm{kJ\,mol^{-1}}$

The enthalpies of reaction [$\Delta_R H_{(\mathrm{calculated})}$] calculated for the modified equations Eq. (5) and Eq. (6), drawn up basing on yield coefficients for biomass and biosurfactant, show good agreement to the enthalpies obtained from calorimetric data [$\Delta_R H_{(\mathrm{experimental})}$]. The calorimetrically determined enthalpies prove as a very useful and suitable measure for the verification of the reaction equations. The equations drawn up regarding the formation of biomass as well as biosurfactant appear as a suitable model for the growth process of *R. opacus* 1CP on the given carbon sources. The data from calorimetric measurement permit the verification of these models and furthermore the monitoring of the growth behaviour even though the culture broth is heterogeneous. A further advantage results from the fact, that if the yield coefficient for biomass and the exchanged heat for the investigated growth process are estimated, the yield coefficient for the biosurfactant formed during the process can be calculated. This often permits to avoid the time consuming biosurfactant quantification by the anthron-sulfuric acid method. A

requirement for this approach is the maintenance of defined growth conditions.

4. Summary

The results of the calorimetric experiments with *R. opacus* 1CP growing on alkanes show, that calorimetric methods are well suited for the investigation of microbial processes even in heterogeneous systems. Calorimetric data presented as power–time curves, displaying the current microbial activity or as enthalpy–time curve, providing the more common integral signal, illustrate the characteristic growth behaviour of the cell culture on alkanes and confirm a good reproducibility of the cultivation. The enthalpy of the growth process proves as a suitable measure for verifying reaction equations for the process. With appropriate reaction equations and a calorimetric monitoring system, product yields can be estimated by calculation from calorimetric data. This is a suitable way for reducing the necessity of time consuming product analytics for instance during optimisation processes when fast results are needed.

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