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ORIGINAL PAPER

Degradation of selected (bio-)surfactants by bacterial cultures monitored by calorimetric methods

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Abstract The subjects of the article are investigations concerning the ability of both *Rhodococcus opacus* 1CP and mixed bacterial cultures to use selected surfactants as sole carbon and energy source. In a comparative manner the biosurfactants rhamnolipid, sophorolipid and trehalose tetraester, and the synthetic surfactant Tween 80 were examined. Particular emphasis was put on a combinatorial approach to determine quantitatively the degree of surfactant degradation by applying calorimetry, thermodynamic calculations and mass spectrometry, HPLC as well as determination of biomass. The pure bacterial strain *R. opacus* was only able to metabolize a part of the synthetic surfactant Tween 80, whereas the mixed bacterial cultures degraded all of the applied surfactants. Exclusive for the biosurfactant rhamnolipid a complete microbial degradation could be demonstrated. In the case of the other surfactants only primary degradation was observed.

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Introduction

Chemical industry manufactures a wide variety of surfactants both for industrial and domestic use. Detergents, paints, adhesives, cosmetics, herbicides, insecticides, and emulsifying agents are common examples for their application. Because of the increasing environmental awareness, the demand for alternative solutions, e.g. microbially produced surfactants, is rising steadily. Due to their biological origin, these so-called biosurfactants are generally assumed to be associated with a lower toxicity and a higher biodegradability, thus, implying a higher environmental compatibility in comparison to their synthetic counterparts (Banat et al. 2000; Kitamoto et al. 2002; Mulligan 2005). However, only few studies are available in the literature dealing with biodegradability and environmental compatibility of biosurfactants. By contrast, the antimicrobial properties of some of them are already well investigated (Haba et al. 2003; Singh and Cameotra 2004).

Poremba and co-workers (1991) investigated growth inhibition of bacteria, microalgae as well as microflagellates by several (bio-)surfactants in seawater medium. Furthermore, the biodegradation of

both synthetic and microbially produced surfactants were studied using fresh seawater as microbial inoculum. The group of Poremba was able to demonstrate that the degradation of the biosurfactants was faster compared to the degradation of their synthetic counterparts, but information is missing regarding the completeness of the biodegradation. Concerning soil bioremediation, comparable investigations with soil microorganisms are scarce or mainly deal with the biodegradability of the well-known rhamnolipids (Czeschka 1995). Rhamnolipids represent the most intensively studied group among the biosurfactants (Maier and Soberón-Chávez 2000; Haba et al. 2003; Mohan et al. 2006). Thus, research groups are often interested in the effect of biosurfactants on the biodegradation of hydrophobic substances, e.g. phenanthrene belonging to the group of polycyclic aromatic hydrocarbons (PAH). In many cases, the biodegradability of the additive is not ensured (Zhang et al. 1997) or the researchers were just able to demonstrate that the applied biosurfactant was not metabolized (Dean et al. 2001; Zhang and Miller 1992).

However, other types of biosurfactants are promising for potential applications, too (Banat et al. 2000; Kitamoto et al. 2002; Mulligan 2005; Singh and Cameotra 2004) and should be the object of further investigations. Of course, the examination of the biodegradability of these substances is one of the prerequisites for their widespread utilization. Moreover, the comparison with the biodegradability of common and perhaps structurally similar synthetic surfactants, whose production might be more cost-efficient, seems to be reasonable. Another aspect is the comparison of the degradability of biosurfactants by both pure bacterial strains and mixed bacterial cultures as it is common in literature concerning synthetic surfactants (Schöberl 1989; van Ginkel 1996). Those experiments would permit a prediction with respect to better biodegradability.

Therefore, the bacterial strain *Rhodococcus opacus* 1CP was employed as pure culture in this study. This bacterium is relevant to such examinations for several reasons: (i) It is a typical soil bacterium that is important for microbial degradation of pollutants in soil. (ii) It produces its own biosurfactant, a trehalose dinocardiomycolate (Niescher et al. 2006), if grown on *n*-alkanes. Therefore the metabolic utilization of similar surfactant molecules by *R. opacus* can be expected. Furthermore, a mixed bacterial culture, obtained by a conventional enrichment technique, was applied for the investigations. The following biosurfactants were used as sole carbon and energy source for the degradation tests: a rhamnolipid from *Pseudomonas aeruginosa*, a sophorolipid from *Candida bombicola* as well as a trehalose tetraester from *Rhodococcus erythropolis* B7g (Fig. 1a–c). Due to the fact that biosurfactants are considered to be an option to common synthetic surfactants, these experiments should be performed and discussed in a comparative way. On this account the synthetic surfactant Tween 80 (Fig. 1d) was additionally used as a model surfactant. This non-ionic surfactant is widely used for soil remediation applications (Kim et al. 2001; Volkering et al. 1998) and the research group of Franzetti et al. (2006) has already shown by means of biochemical oxygen demand (BOD) measurements that Tween 80 is degradable very easily. Furthermore, both the mentioned biosurfactants and the selected synthetic surfactant consist of sugar units linked to hydrophobic alkyl chains by ester or ether bonds. Therefore, some structural similarity is given, providing a basis for the comparison of the degradation of the different surfactants.

For the investigation of the ability of the selected bacterial cultures to use the surfactants as sole carbon and energy source and for the determination of the degree of degradation in this study mainly calorimetric methods were used. Established methods for monitoring the microbial growth, in particular the determination of the optical density (OD), fail due to the observed aggregation of strains like *R. opacus* while growing on hydrophobic substrates (Niescher et al. 2006; Winkelmann et al. 2009) as well due to the formation of foams from the applied surfactants. Especially in this context the advantages of calorimetric measurements become apparent. Because of the non-invasive character and the potentially universal application of calorimetry, it is well suited for the online monitoring of microbial processes. Like any chemical reaction, the growth of microorganisms is associated with heat exchange. That, in turn, is correlated to the metabolized amount of substrate. Thus, our approach of employing the calorimetric measurement along with the determination of the biomass permits a first prediction concerning the progress and the completeness of degradation. In order to introduce the calorimetric method for this

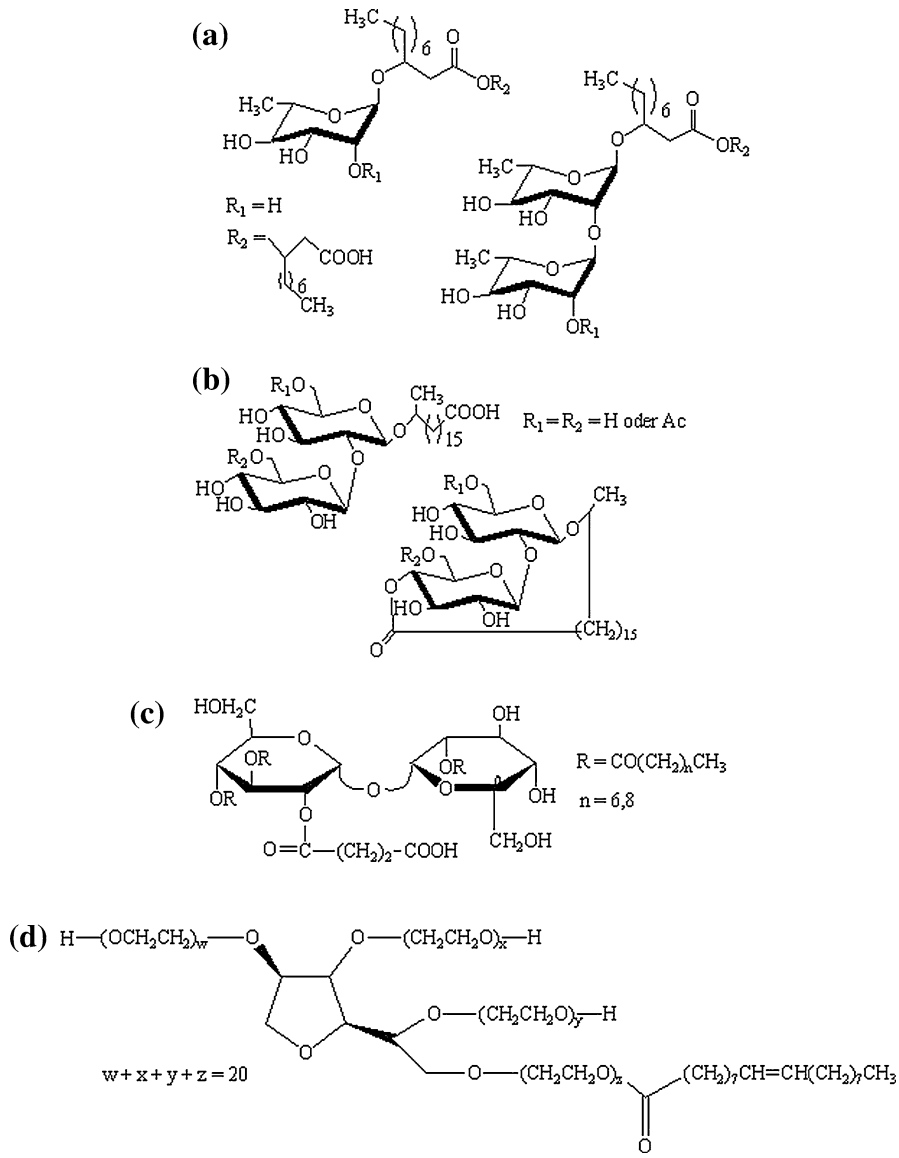

Fig. 1 Structures of the applied (bio-)surfactants: **a** rhamnolipid, **b** sophorolipid, **c** trehalose tetraester, **d** Tween 80

application purpose, a comparison with an established method is given.

Materials and methods

Medium and substrates

The nutrient medium for the degradation tests as well as for the enrichment was a mineral salt medium according to Dorn et al. (1974). One litre of medium contained: 5.6 g Na_2HPO_4, 2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 200 mg $MgSO_4 \cdot 7H_2O$, 40 mg $CaCl_2 \cdot 6H_2O$, 10 mg Fe-ammonium citrate, 300 µg H_3BO_3, 200 µg $CoCl_2 \cdot 6H_2O$, 100 µg $ZnSO_4 \cdot 7H_2O$, 30 µg $MnCl_2 \cdot 4H_2O$, 30 µg $Na_2MoO_4 \cdot 2H_2O$, 20 µg $NiCl_2 \cdot 6H_2O$ and 10 µg $CuCl_2 \cdot 2H_2O$. The medium was amended with the appropriate carbon source. Thus 1 l medium additionally contained: 0.655 g Tween 80 (= 0.50 mmol l^{-1}, M = 1310 g mol^{-1}), 0.975 g rhamnolipid (= 1.50 mmol l^{-1}, M = 650 g mol^{-1}), 0.90 g sophorolipid (= 1.50 mmol l^{-1}, M = 600 g mol^{-1}), 0.80 g trehalose tetraester (= 0.94 $mmol^{-1}$, M = 850 g mol^{-1}), or 0.141 g oleic acid (= 0.5 mmol l^{-1}). The given molecular weights of the surfactants are average values because most of them are mixtures.

The following biosurfactants were used for the investigations: the rhamnolipid-mixture "JBR 425" from *P. aeruginosa* purchased from Jeneil Biosurfactant Company (USA), the sophorolipid "Sopholiance" from *C. bombicola* purchased from Soliance (France) and the trehalose tetraester from *R. erythropolis* B7g. The latter was produced, isolated and purified in the Institute of Biosciences of Technische Universität Bergakademie Freiberg (Frister 2006). The rhamnolipid-mixture "JBR 425" is a 25% aqueous solution of mono- and dirhamnolipid surfactants and the masses and concentrations given above are related to the rhamnolipids themselves, not to the aqueous solution. All the biosurfactants mentioned contain sugar units (rhamnose, sophorose, and trehalose, respectively) connected to alkyl chains by ester or ether bonds. For comparison the synthetic surfactant Tween® 80 (AppliChem, Darmstadt) was used as a model substrate. Tween® 80 is a non-ionic surfactant that contains a sugar unit (sorbitol) linked to four polyether chains as well as to an oleic acid residue acting as hydrophobic part of the molecule. This surfactant is used as emulsifying agent in the food industry.

For additional growth-experiments oleic acid (Merck, Darmstadt) was used as substrate.

Organisms and culture conditions

Rhodococcus opacus 1CP, an aerobic, gram-positive nocardioform actinobacterium was used as pure culture. This organism was originally isolated by its ability to utilize chlorophenols (Gorlatov et al. 1989). Besides the common energy and carbon sources such as glucose numerous long-chain *n*-alkanes (C_{10}–C_{16}) (Niescher et al. 2006), chlorophenols (Moiseeva et al. 1999), methylphenols, 3-chlorobenzoate, and others, are potential substrates. During the growth on long-chain hydrocarbons *R. opacus* 1CP synthesizes biosurfactants. Especially the formation of the glycolipid trehalose dinocardiomycolate has been verified (Niescher et al. 2006). For the experiments the bacterial strain 1CP was cultivated on agar plates (mineral medium containing 5 mM benzoate as carbon source). The colonies were inoculated at regular intervals of approximately 4 weeks. After the incubation time of 2–3 days at 30°C the agar plates were stored at 4°C. In preparation for the calorimetric degradation studies the cultivation of the bacterial strain 1CP as well as the enrichment of the mixed bacterial cultures described below was carried out in shaking flasks on a shaking incubator at 30°C. For the former, 50 ml mineral medium amended with the appropriate (bio-) surfactant was inoculated with a colony of strain 1CP from the agar plates. After the incubation time of 7 days, 50 ml of fresh mineral salt medium was inoculated with 1 ml of the previously obtained bacterial suspension and the related (bio-)surfactant was added. After 5 more days of incubation the cultures were well prepared for the calorimetric degradation experiments. The additional growth experiments with oleic acid as sole carbon and energy source were carried out in an analogous way.

Moreover, four different mixed bacterial cultures were enriched on Tween 80 and on the three biosurfactants. The cultures were obtained from a soil sample from a meadow within the city of Freiberg by a conventional enrichment technique. For this purpose 20 ml of the mineral salt medium described above was inoculated with 0.5 g of the soil and the appropriate (bio-)surfactant was added. First, these cultures were incubated for 14 days at 30°C also in shaking flasks. Afterwards 1 ml of the obtained suspensions was added to fresh mineral salt medium containing the particular (bio-)surfactant (total volume 20 ml). This cycle was repeated seven times over all. Later the incubation times were shortened to 7 days in preparation for the calorimetric degradation studies.

Bacterial growth and related analyses

At the beginning investigations had to be carried out verifying the ability of *R. opacus* 1CP to utilize the (bio-)surfactants as sole energy and carbon source. These experiments were performed using shaking cultures. The measurement of the OD with a spectrometer (Ultrospec 1000, Pharmacia Biotech) at 645 nm was used for growth control of the strain 1CP, except for those experiments in which trehalose tetraester were applied as substrate. Due to the low water solubility of this biosurfactant which interfere optical measurements, thin layer chromatography was employed to show a decrease of this substrate as a result of bacterial growth. For this purpose daily samples of 1 ml of the bacterial suspension were taken over a period of 5 days. The samples were stored at −20°C in Eppendorf tubes. The subsequent treatment included an extraction with 100 µl chloroform (mixing for 1 min on a vortexer and separation

of the phases by 5 min centrifugation at 16,000×*g*). 15–30 µl of the organic phase was applied onto the TLC-plates (covered with silica gel 60 and the fluorescence indicator F254, Merck, Berlin). A mixture of chloroform/methanol/water (65/15/2; v/v/v) served as eluent. A sugar-specific 4-methoxybenzaldehyde/acetic acid/sulphuric acid reagent (0.1/10/0.2; v/v/v) was applied for the derivatization of the separated substances (Kretzschmer et al. 1982). The growth control of the four enriched bacterial cultures was carried out in an analogous manner.

The determination of the dry mass of the produced biomass was performed by a standard method (Bast 1999) as described below. After the calorimetric measurement the whole volume of the culture medium was transferred into 50 ml falcon tubes and centrifuged at 5,000×*g* for 15 min, followed by two washing steps. Afterwards the biomass was resuspended in distilled water, quantitatively transferred into 2-ml-Eppendorf tubes and centrifuged for 10 min at 16,000×*g*. The biomass pellet was dried for 12–18 h at 105°C.

The MALDI-TOF–MS of the pure substrate Tween 80 and of the culture broth after the microbial growth on that surfactant was carried out at the Institute of Macromolecular Chemistry and Textile Chemistry at the TU Dresden with a Biflex IV (Bruker Daltonics) mass spectrometer. Prior to measurement the culture broth was separated from the biomass.

The degradation of oleic acid by *R. opacus* was followed by HPLC (Knauer, Germany). For that, samples of 1 ml of the bacterial suspension were taken every day over a period of 10 days. The samples were stored at −20°C in Eppendorf tubes. The subsequent treatment included an extraction with 100 µl chloroform, separating the organic phase from the aqueous one as well as vaporization of the solvent. Afterwards the sample was redissolved in a mixture of acetonitrile/water acidified with 0.1% acetic acid (95/5, v/v), that also served as the eluent for the HPLC (flow rate 1.0 ml min^{-1}). As stationary phase a 250 × 4.6 mm C_{18}-RP column with a particle size of 5 µm and a pore size of 100 Å was used. Oleic acid was detected after 4.7 min at a wavelength of 205 nm with a UV-detector at ambient temperature.

Calorimetric methods

The metabolic heat release was mainly measured with the calorimeter LKB 8700 (Sunner and Wadsö 1966). The measurements were carried out at 25°C in a 100 ml calorimetric vessel containing 90 ml culture medium, inoculated with 1 ml of the bacterial suspension, prepared as described above. During the experiments the medium was stirred (200 rpm) and aerated with moisturized oxygen (130 ml h^{-1}). Stock solutions of the substrates with the following concentrations were supplied by means of a Hamilton micro-litre syringe: 0.1 mol l^{-1} (Tween 80 as well as rhamnolipid) and 0.012 mol l^{-1} (trehalose tetraester). The supply with the oleic acid was carried out in pure form. The biosurfactant sophorolipid represents an exception in this context. Due to the insufficient solubility of the pure liquid, an adequate dosage by syringe was not possible and, therefore, this substrate was added undiluted enclosed to a glass ampoule into the calorimetric vessel. After an initial time of 24 h, required to achieve the thermal equilibrium, the degradation experiments were started by the addition of the substrate, either by means of the micro-litre syringe or by breaking the ampoule.

Furthermore, the degradation tests with the trehalose tetraester as substrate were carried out using the LKB 2277 Thermal Activity Monitor (TAM, Thermometric AB, Järfälla, Sweden) because of the low water solubility of this (solid) biosurfactant and, consequently, low concentrations of the stock solution (0.012 mol l^{-1}) associated with the demand for a higher sensitivity. For these measurements a 20 ml vessel was filled with 15 ml culture medium, inoculated with 200 µl of the bacterial suspension, prepared as described above. The degradation experiments were started after an initial time of 7 h for thermal equilibration. Other important parameters for microbial growth were similar to the ones used in the LKB calorimeter type 8700 measurements.

Whenever applicable, repeated substrate additions were employed. Precondition for a second substrate addition was the achievement of a constant value of the base line for at least 1 h reflecting no further degradation processes. Mainly, this was given 80–90 h after the first substrate addition.

Results and discussion

This work intends to underline the potential of calorimetric methods for the online-monitoring, the description and estimation of the extend of the

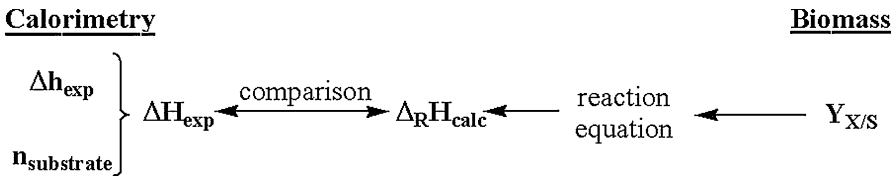

Fig. 2 Scheme of the applied evaluation

microbial degradation process, combined with the determination of only one additional growth parameter—the produced biomass—to make a statement concerning the completeness of biodegradation of substrates like surfactants. For this purpose on the one hand the experimental available enthalpy Δh_{exp} of the entire reaction is required, from which the molar enthalpy ΔH_{exp} is obtained. On the other hand the knowledge of the elemental constitution of the biomass is essential (Winkelmann et al. 2009; von Stockar and Liu 1999) as well as the determination of the produced biomass resulting in the yield $Y_{X/S}$. Thus, a simplified reaction equation can be constructed.

$$C_xH_yO_z + a\ O_2 + b\ NH_3 \rightarrow c\ CH_rO_sN_t + d\ CO_2 + e\ H_2O \quad (1)$$

$C_xH_yO_z$ represents the substrate and $CH_rO_sN_t$ reflects the obtained biomass. Since the microbial growth is governed by complex metabolic processes, the specification of a single reaction equation, representing a simplified equation of anabolic and catabolic processes, is common in the literature (Battley 1999). This approach is sufficient for our purposes, since calorimetric measurements also result in an integral signal including all processes occurring during microbial growth. Hence, the expected molar reaction enthalpy Δ_RH_{calc} was calculated by means of such an equation (Winkelmann et al. 2004). For that purpose the enthalpy of formation for all products and educts in Eq. 1 was required. By means of a theoretical approach according Cordier et al. (1987), introducing the reduction degree γ (number of electrons transferred to oxygen per atom of carbon, Eq. 2) and the coefficient Q (heat evolved per number of available electron equivalents transferred to one gram atom of oxygen during combustion), the calculation of the heat of combustion (ΔH_0, Eq. 3) and thus, the calculation of the enthalpy of formation was possible. Using the applied substrate in Eq. 1 as an example, x, y and z are the stoichiometric indices of C, H and O, respectively.

$$\gamma = 4 + y/x - 2z/x \quad (2)$$

$$Q = 115.06\ kJ \cdot mol^{-1} \quad \Delta H_0 = Q \cdot \gamma \cdot x \quad (3)$$

A simplified illustration of the approach described above is shown in Fig. 2. The scheme depicts the comparison of the results of the calorimetric experiments on the left (Δh_{exp} and ΔH_{exp}, respectively) and the results of the determination of the produced biomass (yield, reaction equation and Δ_RH_{calc}) on the right.

Which conclusions can be drawn from the comparison of the experimentally obtained molar enthalpy ΔH_{exp} and the enthalpy calculated on the basis of the reaction equation as well as the determined biomass (Δ_RH_{calc})? If the values of both are consistent, the complete conversion of the applied substrate according to the given equation can be assumed. By contrast, a difference between the mentioned enthalpies suggests on the one hand an incomplete conversion, i.e. in the case of surfactants, that only a primary degradation took place, on the other hand the formation of secondary metabolites is likely and the reaction equations has to be completed in such cases. However, for the identification of dead end metabolites and certain secondary metabolites further analytical techniques are essential.

Furthermore, for the adequate comparison of the degradability of several substrates differing in the number of carbon atoms within the molecule, the reference to the associated C-mole is recommended, given as the enthalpy ΔH (specified in kJ (C-mol)$^{-1}$ of the applied substrate). However, the general composition of the substrates with respect to the percentage of the amount of oxygen relative to that of carbon within the molecule should be nearly the same. On the basis of the chemical formula normalized to the C-mole (Tween 80: $CH_{1.97}O_{0.41}$, rhamnolipid: $CH_{1.81}O_{0.41}$; sophorolipid: $CH_{1.80}O_{0.40}$, trehalose tetraester: $CH_{1.35}O_{0.40}$) it can be shown, that this precondition is fulfilled for the used (bio-) surfactants.

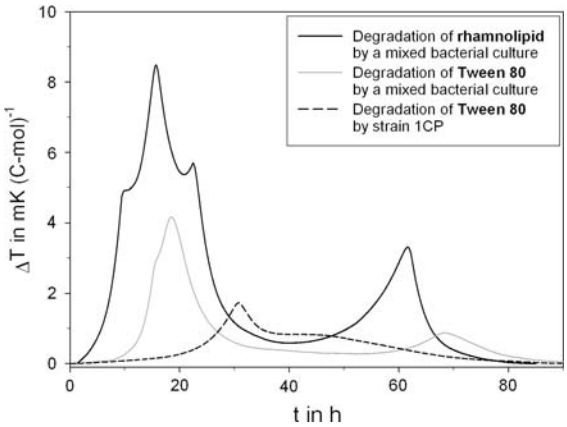

Fig. 3 Calorimetric growth curves of the degradation of rhamnolipid by a mixed bacterial culture as well as of the degradation of Tween 80 by both a mixed bacterial culture and *R. opacus* 1CP (first substrate addition). For the cultivation mineral salt medium was applied. The experiments were carried out at 25°C. Each experiment was started by the addition of 0.09 mmol surfactant (= 1.0 mmol l^{-1}) as sole carbon and energy source

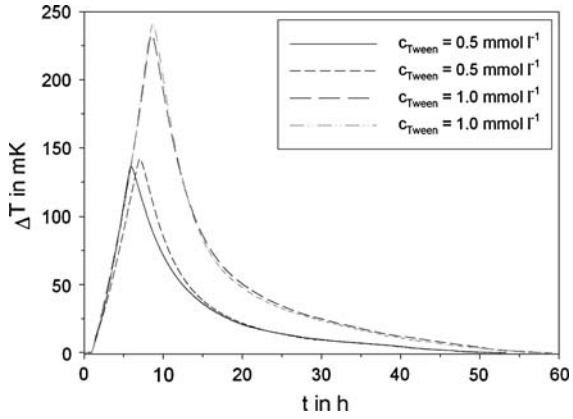

Fig. 4 Calorimetric growth curve of *R. opacus* 1CP in mineral salt media on Tween 80 (second substrate addition). The experiments were carried out at 25°C and were started with a substrate addition by micro-litre syringe. The achieved high reproducibility is clearly shown

Degradation of Tween 80 by *Rhodococcus opacus* 1CP

Prior to the degradation experiments in the calorimeter, pre-cultures of *R. opacus* were grown with Tween 80 as shaking cultures and prepared for the calorimetric measurements as described above. After the first substrate addition a relatively long lag-phase was observed (see Fig. 3, degradation of Tween 80 by *R. opacus*, dashed lines), although *R. opacus* was expected to be sufficiently adapted to the substrate. Possibly the loss of microbial activity (i.e. enzyme activity) was caused by the long time of 24 h required for the thermal equilibration of the calorimeter LKB 8700. Furthermore, the adaptation to the somewhat different cultivation conditions could be responsible for that, e.g. the cultivation of the shaking cultures was carried out at 30°C, whereas the measurements in the calorimeter took place at 25°C.

However, for the validation of the adequate adaptation of *R. opacus* to the conditions inside the calorimeter, the calorimetric curves obtained after the second substrate addition were used for the evaluation. Thus, high reproducibility of the molar enthalpy was achieved, independent from the applied amount of substrate. As shown in Fig. 4 there was obviously no noteworthy lag-phase. Therefore, the immediate start of the presented measurements reflects the expected adaptation to the given culture conditions and, furthermore, it shows that, in the applied concentrations, Tween 80 does not seem to be toxic to *R. opacus*. The whole degradation process required almost 60 h.

For the growth of *R. opacus* on Tween 80 a molar enthalpy of $\Delta H_{exp} = -(6000 \pm 300)$ kJ mol^{-1} was determined. Furthermore, the analysis of the produced biomass gave a yield of $Y_{X/S} = (0.09 \pm 0.01)$ g g^{-1}. From this value the following reaction equation could be derived:

$$C_{64}H_{126}O_{26} + 75.93\ O_2 + 0.61\ NH_3 \rightarrow 5.5\ CH_{1.85}O_{0.37}N_{0.11} + 58.5\ CO_2 + 58.82\ H_2O \quad (4)$$

Assuming this equation for the conversion of Tween 80 ($C_{64}H_{126}O_{26}$) into biomass (the elemental composition was determined in previous work, Winkelmann et al. 2009), water and carbon dioxide, a molar reaction enthalpy of $\Delta_R H_{calc} = -34940$ kJ mol^{-1} would be expected. The comparison with the calorimetrically determined molar enthalpy ($\Delta H_{exp} = -6000$ kJ mol^{-1}) shows a rather large difference suggesting the incomplete conversion of Tween 80.

In order to validate the incomplete degradation in the first instance and additionally to identify the remaining fragments, mass spectra of the pure substrate Tween 80 and of the culture broth after the microbial growth were taken. As shown in Fig. 5, both mass spectra contain fragments typical for ethoxyl groups ($\Delta m = 44$ m/z), representing the

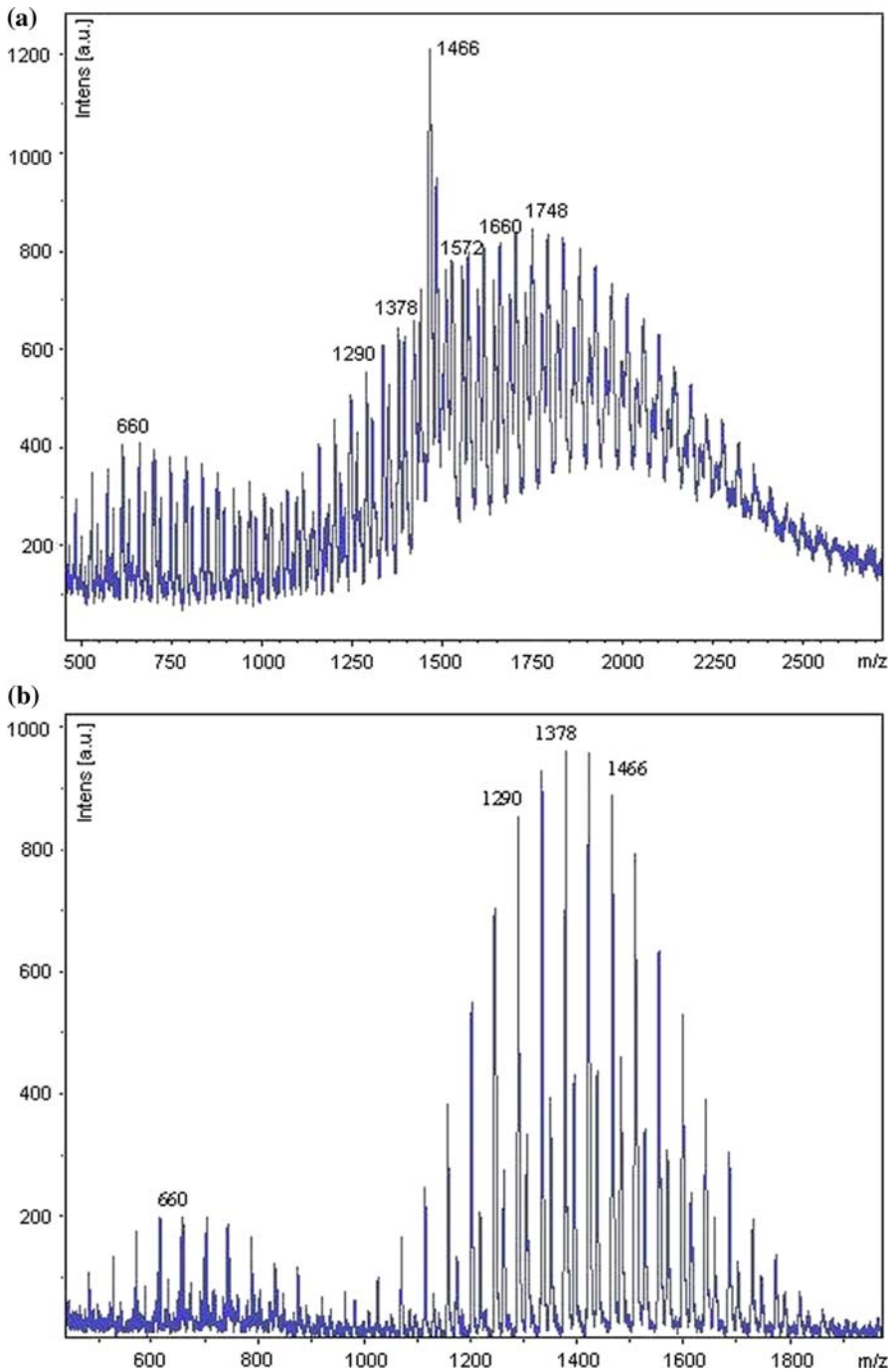

Fig. 5 Mass spectra **a** of the pure substrate Tween 80 and **b** of the culture broth after microbial growth (separated from the biomass). Both spectra contain fragments with $\Delta m = 44$ m/z representing the ethoxyl groups of the surfactant. However, spectrum **b** (culture broth) shows a shift to lower m/z ratios compared to spectrum a (pure substrate), reflecting the degradation of at least one part of the surfactant molecule

hydrophilic part of the surfactant (see also Fig. 1d). Consequently, this structural moiety does not seem to be converted by *R. opacus*. The mass spectrum of the culture broth, however, shows a shift to lower ratios of m/z (see Fig. 5b) indicating the degradation of at least one fragment. Hence, the hydrophobic alkyl chain corresponding to oleic acid must have been utilized. Furthermore, the employed bacterial strain is known for its ability to metabolize long-chain *n*-alkanes as C-sources. Due to these results and in

Table 1 Results of the degradation of several substrates by *R. opacus*

C-source	ΔH_{exp} (kJ mol^{-1})	ΔH (kJ (C-mol)$^{-1}$)
n-Tetradecane $C_{14}H_{30}$[a]	−3766	−269
n-Hexadecane $C_{16}H_{34}$[a]	−4690	−290
Oleic acid $C_{18}H_{34}O_2$	−5000	−280
Tween 80 $C_{64}H_{126}O_{26}$[b]	−6000	−94

[a] Winkelmann et al. (2009)

[b] Separation in respect to the variation in the C:O ratio

order to verify this hypothesis, additional investigations were carried out applying oleic acid as sole carbon and energy source.

Rhodococcus opacus is able to grow on oleic acid, but the degradation required about 90 h. The increase of the degradation time compared to the degradation of Tween 80 by *R. opacus* is most likely caused by the very low water solubility and the resulting low bioavailability of this hydrophobic substrate. Nevertheless, the determination of the molar enthalpy for the growth of *R. opacus* on oleic acid resulted in the same order of magnitude as on Tween 80 (see also Table 1). Thus, the bacterial strain 1CP is able to grow on oleic acid, resulting in a similar molar enthalpy as during growth on Tween 80 and, therefore, suggesting that the values of the molar enthalpies refer to the degradation of the same chemical structure. Additional HPLC analyses of the oleic acid content from samples taken during microbial growth confirmed that the applied oleic acid was completely metabolized by *R. opacus*. After 3 days almost all of the substrate was converted agreeing with the calorimetric experiments, and after 4 days the substrate was no longer detectable by HPLC. These arguments support the established hypothesis that only the oleic acid residue from the surfactant molecule had been degraded by *R. opacus* while growing on Tween 80.

Degradation of the selected (bio-)surfactants by mixed bacterial cultures

Rhodococcus opacus 1CP was not able to utilize the supplied rhamnolipid, sophorolipid, and trehalose tetraester. Therefore, similar degradation experiments were repeated by means of mixed bacterial cultures. For that purpose, conventional liquid enrichment cultures were prepared, in which the above biosurfactants as well as the synthetic surfactant Tween 80 were supplied in mineral medium and served as sole sources of energy and carbon. Soil was used as an inoculum and growth (30°C) was determined by OD measurements. Cultures, obtained after seven transfers from the initial enrichments in fresh medium, were used for calorimetric investigations. Microscopic analysis of the four pre-cultures indicated the presence of morphologically similar phenotypes; however, a correlation of this observation with the dominance of certain specialized degraders has not been investigated.

Depending on its rate of degradation, the calorimetric behaviour of each (bio-)surfactant was followed up to 90 h. Differently shaped, but otherwise highly reproducible curves were the results of these measurements whose integrals were used for the calculation of the molar enthalpies of the degradation of the biosurfactants. A summary of the results is given in Table 2. Typical curves for the degradation of rhamnolipid as well as Tween 80 by mixed bacterial cultures obtained by calorimetry are shown in Fig. 3. The depicted measurements were initiated with low biomass concentration and an amount of 0.09 mmol substrate was added to start each of the experiments. For the growth of the mixed bacterial cultures on Tween 80 a somewhat longer lag-phase compared to the degradation of rhamnolipid was noticeable, but the growth rate in the exponential phase seemed to be very similar. On the other hand the calorimetric curves differ in the height of the signals.

Table 2 Results of the degradation of the (bio-)surfactants by mixed bacterial cultures

(Bio-)surfactant	ΔH_{exp} (kJ mol^{-1})	ΔH (kJ (C-mol)$^{-1}$)	$Y_{X/S}$ (g g^{-1})	$\Delta_R H_{calc}$ (kJ mol^{-1})	Degradation
Tween 80 $C_{64}H_{126}O_{26}$	−9400	−150	0.045	−36720	Primary
Rhamnolipid $C_{32}H_{58}O_{13}$	−12700	−400	0.42	−12655	Total
Sophorolipid $C_{30}H_{54}O_{12}$	−4400	−150	0.20	−14370	Primary
Trehalose tetraester $C_{43}H_{58}O_{17}$	−8720	−200	0.11	−20090	Primary

As shown in Fig. 3 the temperature difference, which is correlated with the heat release during growth (here normalized to C-mole of the applied substrate), for the degradation of rhamnolipid was almost twice that for the degradation of Tween 80. This distinction obviously results from the different degree of degradation of the examined (bio-)surfactants. Interestingly, the characteristics of rhamnolipid degradation measured by calorimetry clearly indicated the splitting up of the heat signal into four distinguishable peak maxima. Since every peak maximum should reflect a certain time-limiting reaction or process, calorimetry here gives first evidence for the existence of several "bottlenecks" in the degradation pathway of rhamnolipids. These are either the result of a retarded degradation of structural moieties within one microorganism or reflect syntrophic activities between different species. By contrast, the degradation of Tween 80 exhibits only two distinctive peak maxima. The integral of the first peak ($\Delta H = -(96 \pm 9)$ kJ $(C\text{-mol})^{-1}$) is comparable to the result of the degradation of Tween 80 by *R. opacus* ($\Delta H = -94$ kJ $(C\text{-mol})^{-1}$). Therefore, the oleic acid moiety of Tween 80 seems to be converted by the mixed bacterial culture in the first step, as well. Aside from that, the mixed bacterial culture obviously was able to metabolize another part of the surfactant. The structure of it was not further analysed. Nevertheless, the occurrence of more than one peak maximum seems to be characteristic for the degradation of such complex molecules as the applied surfactants by mixed bacterial cultures. This is also supported by the calorimetric curves of the degradation of the other two biosurfactants by the mixed bacterial cultures. In Figs. 6 and 7 the growth on sophorolipid and trehalose tetraester, respectively, is shown. For both the growth after the second substrate addition during the calorimetric measurement is depicted. The sophorolipid was added undiluted enclosed to a glass ampoule into the calorimetric vessel and the degradation experiments were started by breaking the ampoule. The obtained growth curves reveal broad peaks and denoting a shoulder that also reflects more than one degradation steps. Due to the low concentrations of the stock solution of the trehalose tetraester, demanding a higher sensitivity of the calorimeter, for these degradation experiments the TAM was used. The calorimetric growth curve is here given by means of the heat power, likewise two distinct peak maxima are noticeable. Hence, for monitoring such effects, calorimetry is well suited. Therefore, this method is able to reveal diverse degradation steps and to identify characteristic points within the degradation pathway that are interesting for further analytical studies.

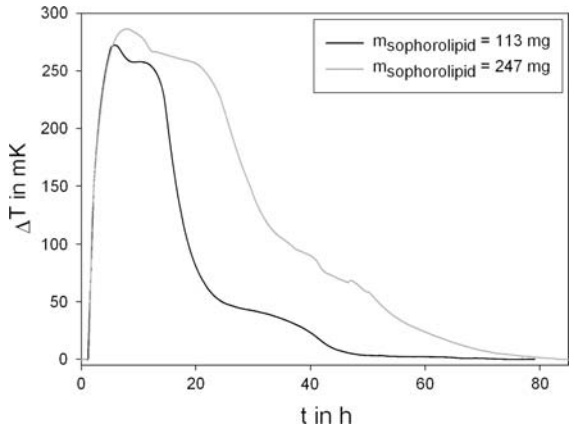

Fig. 6 Calorimetric growth curves of the degradation of sophorolipid by a mixed bacterial culture (second substrate addition). For the cultivation mineral salt medium was applied. The experiments were carried out at 25°C. The given masses of the sophorolipid are equal to 0.19 and 0.41 mmol surfactant (2.1 and 4.6 mmol l^{-1}, respectively). Each experiment was started by the addition of the substrate by breaking the glass ampoule

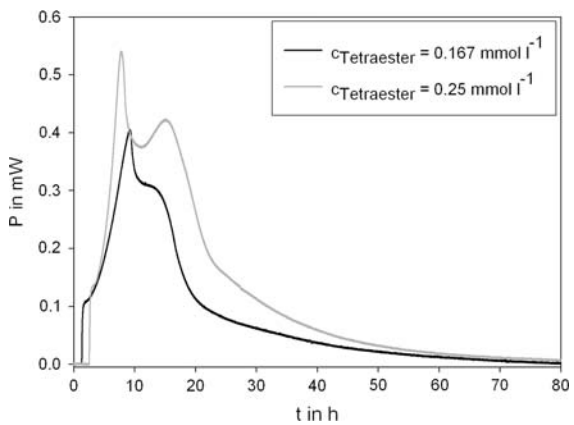

Fig. 7 Calorimetric growth curves of the degradation of trehalose tetraester by a mixed bacterial culture (second substrate addition). For the cultivation mineral salt medium was applied. The experiments were carried out at 25°C and were started with a substrate addition by micro-litre syringe. These degradation experiments were performed in the Thermal Activity Monitor (TAM)

A comparison of the degree of degradation of the examined (bio-)surfactants shows that complete utilization was restricted to rhamnolipid. The integral of

the different peak areas (Fig. 3), representing the different degrees of degradation, is related to the molar enthalpies given in Table 2. The evaluation of the degradation of the biosurfactant rhamnolipid thus exemplifies the potential of the introduced combination of methods. For the growth of the mixed bacterial culture on rhamnolipid as sole carbon and energy source a molar enthalpy of $\Delta H_{exp} = -(12700 \pm 400)$ kJ mol^{-1} was determined. The examination of the produced biomass gave a biomass yield of $Y_{X/S} = (0.42 \pm 0.03)$ g g^{-1}. Corresponding to this yield and in consideration of the elemental composition of the biomass (average value for bacteria, von Stockar and Liu 1999) the following reaction equation was established:

$$C_{32}H_{58}O_{13} + 27.59\ O_2 + 2.48\ NH_3 \rightarrow 11.8\ CH_{1.66}O_{0.41}N_{0.21} + 20.2\ CO_2 + 22.92\ H_2O \quad (5)$$

This reaction equation leads to an enthalpy of $\Delta_R H_{calc} = -12655$ kJ mol^{-1}. Comparing the values of the calorimetrically obtained enthalpy ($\Delta H_{exp} = -12700$ kJ mol^{-1}) and the enthalpy $\Delta_R H_{calc}$, calculated from the biomass yield, just a slight deviation (<1%) is noticeable. The almost perfect agreement of ΔH_{exp} and $\Delta_R H_{calc}$ suggests that the mixed bacterial culture was not only able to utilize the rhamnolipid as sole carbon and energy source, but also to degrade this biosurfactant completely.

Conclusion

The ability of a pure culture (*R. opacus*) as well as four mixed bacterial cultures to use selected surfactants as sole carbon and energy source was investigated in a comparative manner. To provide an adequate basis for the discussion of the different degree of degradation of the applied (bio-)surfactants the enthalpy, normalizing the experimental enthalpy to the associated C-mole (Table 2), was introduced.

Rhodococcus opacus as a pure culture was only able to utilize the synthetic surfactant Tween 80, and based on the measured heat release, just a primary degradation was noticeable. The corresponding enthalpy was $\Delta H = -(94 \pm 5)$ kJ (C-mol)$^{-1}$. Compared to the theoretical enthalpy, derived from the experimentally determined biomass ($\Delta_R H_C = -550$ kJ (C-mol)$^{-1}$); the former value is rather low and, therefore, argues for the incomplete degradation of Tween 80 by *R. opacus*. Comparison of mass spectra of the substrate and of the culture broth after microbial degradation led to the assumption, that only the hydrophobic part of the surfactant corresponding to oleic acid has been converted. Further investigations applying oleic acid as sole carbon and energy source were in agreement with this hypothesis. In this case the enthalpy was $\Delta H = -(280 \pm 17)$ kJ (C-mol)$^{-1}$. Comparable results are known from previous investigations, examining the degradation of long-chain *n*-alkanes such as *n*-tetradecane and *n*-hexadecane by the same pure culture (Winkelmann et al. 2009). In that study molar enthalpies of $\Delta H = -269$ kJ (C-mol)$^{-1}$ for *n*-tetradecane and $\Delta H = -290$ kJ (C-mol)$^{-1}$ for *n*-hexadecane (Table 1) have been determined. Furthermore, it has been proven that the applied substrates were completely metabolized, while *R. opacus* simultaneously produced its own biosurfactant. Since the result of this work resulted in a similar value for the molar enthalpy, the formation of biosurfactants by *R. opacus* while growing on oleic acid is reasonable.

By contrast, the pure culture of *R. opacus* was not able to degrade the examined biosurfactants (in the applied concentrations of up to 1.0 mmol l^{-1}) that have been claimed to be better biodegradable than their synthetic counterparts (Kitamoto et al. 2002; Poremba et al. 1991). However, this result is consistent with the studies of Dean et al. (2001) and Zhang and Miller (1992) demonstrating that there is no compulsion that e.g. rhamnolipid produced by *Pseudomonas* sp. is degradable by other *Pseudomonas* strains or even is degradable by its own "producer-"strain.

The mixed bacterial cultures enriched from a soil sample were able to use all of the applied surfactants as sole carbon and energy source, but only the biosurfactant rhamnolipid could be degraded completely. The results of the investigations of the degree of degradation of the other selected (bio-) surfactants suggest that only primary degradation took place. Even the mixed bacterial cultures, that should have been adapted to the respective (bio-) surfactant as sole carbon and energy source, were not able to degrade these substances completely. Furthermore, only primary degradation of the synthetic surfactant Tween 80 was observable employing the

mixed bacterial culture. Compared to the work of Franzetti et al. (2006), demonstrating the complete degradation of this synthetic surfactant, discrepancies are evident. That illustrates the problem of using different soil samples which naturally are composed of a somewhat other microbial community. Therefore, the distinction between two strategies is essential: (i) A variety of soil samples, activated sludge etc. is used to optimize the microbial degradation striving for complete surfactant degradation and maybe the detailed identification of the subsequent degradation steps. (ii) Using the same soil sample as microbial source should reveal a tendency of the degree of surfactant degradation in the first instance. The mentioned calorimetric measurements pursued the second strategy with the aim of the evaluation of the degree of degradation in a comparative manner especially with respect to synthetic and biosurfactants. Hence, without further information direct comparisons with other works are inapplicable.

However, the degree of degradation of Tween 80 by the pure culture of *R. opacus* was comparable to the first part of the conversion of this surfactant by the mixed bacterial culture. Calculations for both show that most likely the oleic acid residue was metabolized. Hence, this structural moiety seems to be converted very easily by microorganisms. By contrast, the sorbitol ring linked to four polyether chains was more persistent, which is probably caused by the ether bonds which are relatively stable.

The degree of degradation is also reflected by the enthalpy per C-mole. For primary degradation enthalpies of about $\Delta H = -150$ kJ $(\text{C-mol})^{-1}$ and $\Delta H = -200$ kJ $(\text{C-mol})^{-1}$, respectively, were obtained. The total degradation of the rhamnolipid yielded an enthalpy of $\Delta H = -400$ kJ $(\text{C-mol})^{-1}$. Hence, with few prerequisites as described above, the enthalpy per C-mole is a significant quantity to estimate the degree of degradation. Furthermore, calorimetry is a powerful tool for identifying characteristic points within the degradation pathway of complex molecules. These indications may be relevant for further analytical investigations.

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