The Boundary Layer as Reaction Compartment for the Synthesis of Hydroxyalkyl-functionalized Siloxanes

Peter Fröhlich and Martin Bertau
Institute of Chemical Technology, Freiberg University of Mining and Technology, 09596 Freiberg (Sa.), Germany

Reprint requests to Professor M. Bertau. Fax: +49 3731 39-2324. Tel.: +49 3731 39-2384.
E-mail: martin.bertau@chemie.tu-freiberg.de

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Dedicated to Professor Heribert Offermanns on the occasion of his 75th birthday

Enzyme catalysis in two-phase systems, particularly in the functionalization of siloxanes, is a new challenge for biocatalysis. On the basis of three different reaction systems, stirred tank reactor, enzyme membrane reactor and micro mixer, the influence of the phase boundary layer along the interface between an aqueous system and an organosiloxane on the synthesis of hydroxyalkyl-functionalized siloxanes is discussed and solutions presented. Micro mixers produce, through a large surface area to volume ratio, boundary layers within which through a formate dehydrogenase-catalyzed reaction the conversion of 1,3-bis-(3-formoxypropyl)tetramethyldisiloxane to 1,3-bis-(3-hydroxypropyl)tetramethyldisiloxane can be achieved. In contrast, in a stirred tank reactor even after 96 h only 45 % of the formoxy siloxane has been converted.

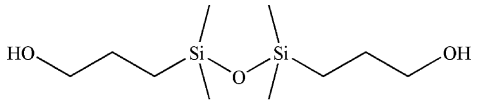
Key words: Enzymes, Siloxanes, Protection Group

Introduction

Organosiloxanes bear flexible Si–O backbones which are functionalized terminally (Fig. 1). This interplay between the properties of siloxane and organic moieties determines the unique chemical and physical properties of the respective systems.

Typical fields of application are personal care products, pharmaceuticals, dyes, varnishs, and coatings as well as building materials. Among the $\alpha,\omega-$hydroxy-functionalized siloxanes particularly hydroxyterminated alkyl siloxanes exhibit excellent adhesion properties for which they are used as additives in adhesives, washing agents and detergents, and for surface modification to be used for instance in membranes permeable for gases as shown in Fig. 2 [1, 2].

A common method for the synthesis of hydroxyalkylsiloxanes proceeds through the addition of silicon hydrides to olefinic compounds with hexachloroplatinic acid as catalyst [3, 4]. On an industrial scale, polymerization of hydroxyalkyl-functional siloxanes is catalyzed by sulfuric acid under equilibration of 1,3-bis(3-hydroxypropyl)tetramethyl disiloxane (**1**) with octamethyltetrasiloxane (Fig. 3).



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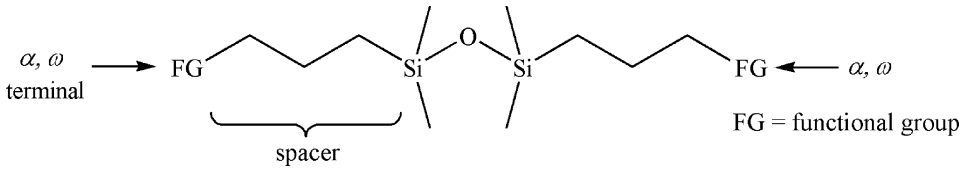

Fig. 1. Organosiloxanes bearing terminal functions.

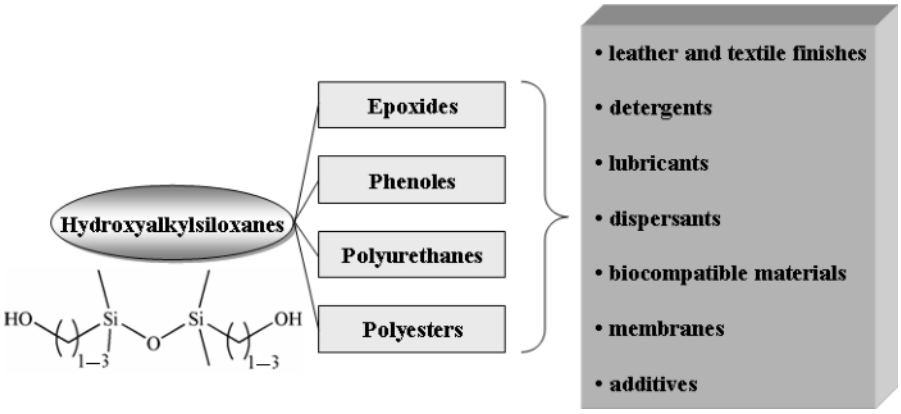

Fig. 2. Hydroxyalkylsiloxanes are important intermediates in siloxane chemistry for extensive applications.

A disadvantage of this method is the poor distillative performance of polysiloxanes with different chain length, and otherwise the low selectivity of the sulfuric acid which often leads to chain termination in the final product. This entails a broadening of the molecular weight.

The main disadvantage of this process is the high reactivity of the carbinol entity which leads to attacks at the silicon atoms resulting in intramolecular ring closure. The inclination of hydroxyalkylsiloxanes for that kind of self-depolymerization is called "backbiting" and demonstrated in Fig. 4 [5].

Thus the aim of the present study was a more selective protocol than the current production process for these organosiloxanes to attenuate the carbinol reactivity and prevent the backbiting. Formates are already known as protective groups in organic synthesis. However, the hydroxyalkyl entity of **1** has to be released by an easy deprotection step of 1,3-bis-(3-formoxypropyl)tetramethyl disiloxane (**2**). This step requires a mild reagent to prevent further chain degradation. Biocatalysts, especially enzymes, exert their catalytic activity under mild and physiological reaction conditions with high selectivity in contrast to the established chemical methods (Fig. 5).

As will be shown below, micro reaction technology can help to evade the frequently encountered limitations of biocatalytic processes in water-immiscible media. In principle, enzymatic reactions in organic media and in silicones have been established [6]. Another area of application are two-phase systems, where the starting material is only slightly soluble in water.

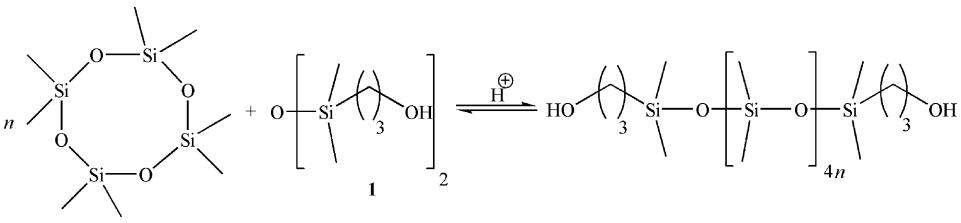

Fig. 3. Polyhydroxyalkylsiloxanes are synthesized by equilibriation-polymerization.

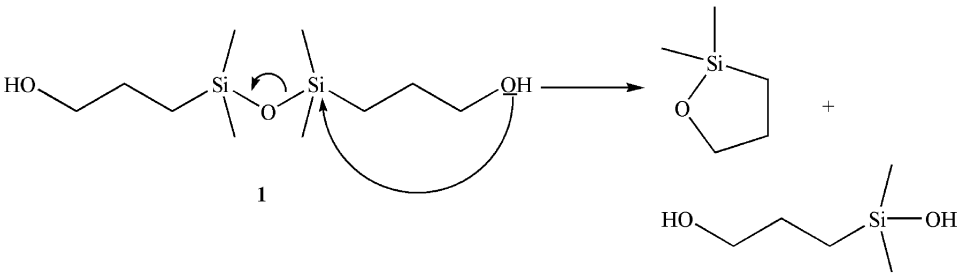

Fig. 4. Intramolecular degradation of **1**.

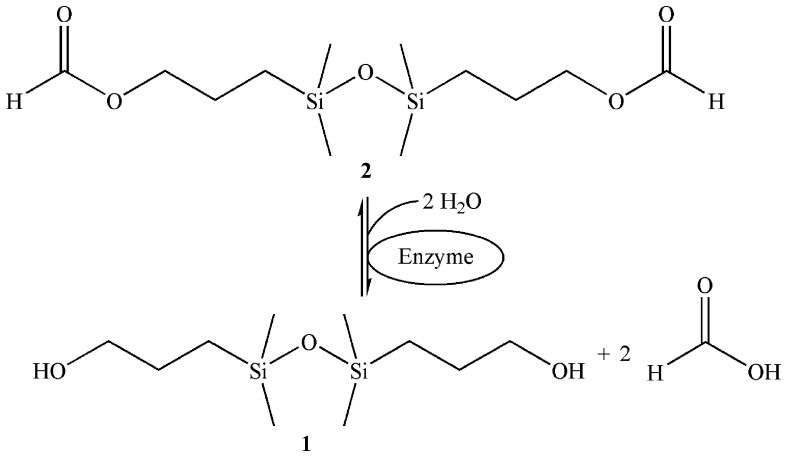

Fig. 5. Enzymatic deprotection of **2**.

These strategies apply not only to organic solvents, they also include ionic liquids or silicone phases. Typically, these biocatalytic reactions proceed diffusion-controlled at the phase boundary owing to the generally very low water solubility of the organic feedstock. However, it is not clear what the implications are if the reactant is not only miscible with water, or if the reactant is the water-immiscible phase itself. An instructive example of how the mass transport along the boundary layer can be significantly improved through the use of microstructured systems is therefore presented in this contribution exemplified by the enzyme-catalyzed production of α,ω-bis-(3-hydroxypropyl) tetramethyldisiloxane (**1**) as a model siloxane.

Experimental Section

General

Enzymes and NAD^+ were kindly provided by Julich Chiral Solutions GmbH, a Codexis company. Other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA). The reagents and solvents were reagent-grade and used as received without further purification.

Instruments

All reactions were monitored by gas chromatography (GC), infrared (IR) and ^{1}H NMR spectroscopy. Quantitative GC analyses were conducted using a Clarus 500 gas chromatograph from Perkin Elmer Inc. (Waltham, MA, USA). The multilamination mixer was provided by Ehrfeld Mikrotechnik BTS GmbH.

*Cleavage of formate **2***

Esterase PL (pig liver), Esterase CLEA (*Escherichia coli*), Esterase BS2 (*Bacillus subtilis*), Esterase P2 (*Pseudomonas fluorescens*), lipase CA (*Candida antarctica*), and Lipase C1 (*Candida cylindracea*) were used in the assays.

0.1 % – 1 % (v/v) of each enzyme was dissolved in 10 mL of 50 mM phosphate buffer, and the reaction was started by adding 10 mmol of **2** dissolved in 800 mL of *n*-hexane. The mixing was performed in a rotary shaker at room temperature. The reaction was monitored by IR spectroscopy by integration of the carbonyl band at 1745 cm^{-1} and the hydroxy band at 3300 cm^{-1}.

In addition to hydrolases, formate dehydrogenase FDH-CB (*Candida boidinii*) was used for the cleavage of **2**. The medium consisted of a mixture of phosphate buffer and isopropanol in the same volume ratio. Therein 125 μL of a 10 mM NAD^+ solution was dissolved in portions, **2** was added and the mixture stirred until the turbidity had disappeared. By adding 10 μL of FDH-CB the reaction was started, and the course of the reaction was followed by gas chromatography.

Table 1. Parameters for the analysis of **2** by gas chromatography.

Parameter	Settings
Column	DB-35 ms (Agilent Technologies)
Injection temp.	250 °C
Detection temp.	260 °C (FID)
Flow; split	1 mL min^{-1} (H_2), 80
Temp. prog.	50 °C → 5 K min^{-1} → 150 °C → 10 K min^{-1} → 250 °C

Conversion within the enzyme membrane reactor (EMR)

The EMR was used in continuous mode and in recirculation mode. The system consisted of two dosing pumps which separately transported the enzyme suspended in buffer by 0.1 % – 1 % (v/v) and the substrate **2** in a pressure-stable stainless-steel cell. The ultrafiltration membrane had a nominal molecular weight cutoff of 10,000 Da. The filtrate passed a separator where the aqueous and the organic phase were separated, and the siloxane phase was recirculated and refilled with fresh buffer. The reaction temperature was 25 °C, and the volume flow per dosing pump was 1 mL min^{-1} at an operating pressure of 1.3 – 1.5 bar. The reaction time was 24 h based on gas chromatographic analysis of conversion of **2** and formation of the hydroxyalkylsiloxane **1** using 1-octanal as internal standard. The parameters for detection are shown in Table 1.

Conversion within the micro reactor

The micro reaction system consisted of two tanks in which the enzyme and the substrate were stored separately. The enzyme and the substrate were pumped *via* a dosing pump together with the substrate **1** at a total flow rate of 0.5 – 3 mL min^{-1} into a multilamination mixer. Toluene and TBME were used as solvents. The slit plate widths of the lamellae were 25 μm, 50 μm and 100 μm and the width of the diaphragm plates 25 μm. Variable temperatures in the range of 20 – 90 °C were adjusted by two micro heat exchangers. The downstream was flowing into a loop with a length of 50 cm (ID = 1000 μm) to prolong the reaction time and for partial de-emulsification. In discontinuous operation mode the product stream was heated up to 90 °C for enzyme denaturation to prevent further reactions. In continuous operation the product stream was circulated in the storage tank. The analysis of **2** was performed by gas chromatography.

Results and Discussion

*Hydrolase activities towards **2***

Because the formoxy-protected siloxane constitutes no natural substrate for hydrolases, the measured enzyme activities range one to three orders of magnitude lower than the corresponding enzyme activities with the standard substrates. The enzyme activities are summarized in Table 2.

Pig liver esterase (PLE) which is frequently used in organic synthesis due to its low substrate specificity showed the highest activity for the hydrolysis of **2**. Among the two lipases tested, with 53.4 mU mg^{-1} lipase C1 displayed the highest activity. As will be shown below, the apparently lower activity of CAL B towards **2** is not attributable to a different structure of the active site compared to lipase C1, even though this interpretation appears reasonable on first sight, since Jaeger *et al.* and Ollis *et al.* had shown that different α/β-hydrolase folding patterns result in altered chemical environments which are known to exhibit different activities towards a substrate [7, 8].

Table 2. Hydrolase activities for substrate **2**.

Enzyme	Activity towards **2** as substrate in mU mg^{-1}
Esterase CLEA	10.0
Esterase BS2	5.8
Esterase P2	10.0
Esterase PL (PLE)	51.3
Lipase C1	53.4
Lipase CA (CAL B)	8.4

*Reactor concepts for the deprotection of **2***

Stirred tank reactor (STR)

In STR experiments the tested enzymes were shown to be active up to 96 h. The best results were obtained with PLE for which 35 % conversion were observed after 96 h. Although this value is far away from completion, it has to be considered that **2** does not correspond to the general substrate spectrum of all enzymes investigated. Moreover, hydrolase-mediated hydrolysis of formic acid esters has been the subject of only very few literature reports, with the spectrum of use remaining limited to *N*-formylated peptides or metabolic breakdown products in methylotrophic microorganisms [9 – 11]. However, in view of the long reaction times and the rather negligible effects that stirring rates had on conversion, further attempts to improve the STR-process were cancelled. It soon became apparent that deep mixing of the two immiscible phases is a prerequisite which cannot be fulfilled without mechanically denaturing the biocatalyst. Since on the other hand the enzyme only enhances establishment of the reaction equilibrium between fully protected, half deprotected and fully deprotected species, it is clear that re-esterification is getting more important with increasing residence time, if formic acid is not removed from the system.

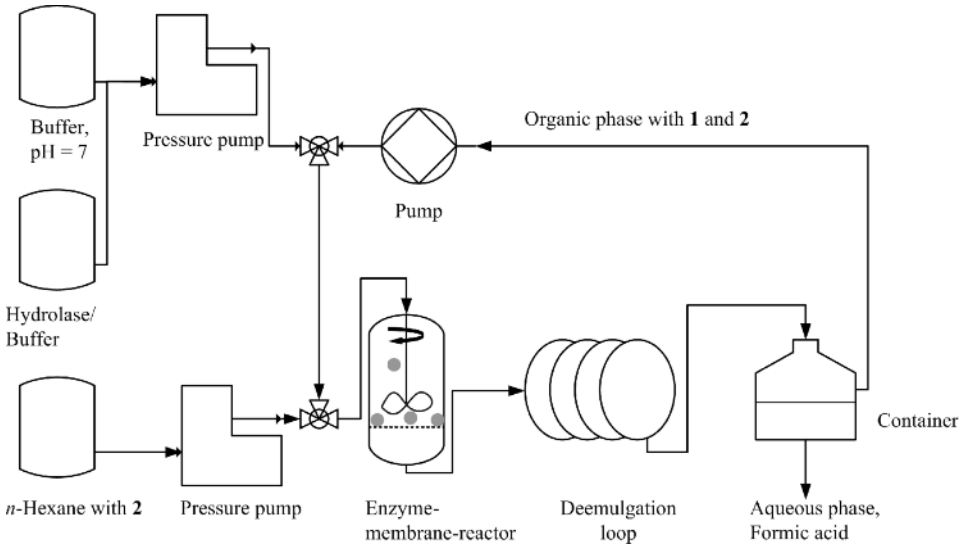

Fig. 6. EMR with continuous separation of formic acid.

Enzyme membrane reactor

Consequently an option to shift conversion to the product was seen in continuously removing the formic acid released from **2**. For these purposes an enzyme membrane reactor (EMR) appeared as the ideal choice, because this reactor allows for holding back the enzyme while formic acid can be separated from the reaction mixture after passing an ultrafiltration membrane (Fig. 6). In addition, incompletely converted reaction mixtures can be recirculated easily. However, one drawback consisted in the necessity to employ *n*-hexane as a solvent in order to reduce filtration resistance along the ultrafiltration membrane. Applying pure siloxane results in almost complete loss of activity, with a residual activity of only 7 % after 120 min due to the high shearing forces. At the same time there is too little reaction mixture passing the membrane what would result in uncompetitively low space-time-yields.

With the flow rate ranging between 1 – 2 mL min^{-1} and an operating pressure of 0.1 – 0.3 MPa the resulting emulsion passed the membrane easily. The membrane exclusion size of 10,000 Da allowed for retaining the hydrolase effectively. The emulsified reaction mixture de-emulsified completely within 10 min in the loop installed downstream, what considerably facilitated formic acid removal. The aqueous phase was separated, and the withdrawn volume was continuously replaced by fresh buffer solution, while the organic phase was recirculated. Applying this setup, enzyme losses were kept < 1 %, and the reaction equilibrium was shifted in favor of the hydroxyalkylsiloxane product reaching 61 % conversion compared to 45 % in the STR (Fig. 7).

With reaction times less than 24 h, effects of formate on catalyst activity are low due to its low concentration. When reaction times exceed 24 h, however, the formic acid concentration increased in the STR leading to reduced turnover rates owing to two effects: firstly an inhibition of CAL B and secondly, a shift of the chemical equilibrium to the educt. A pH-induced enzyme deactivation was excluded, because

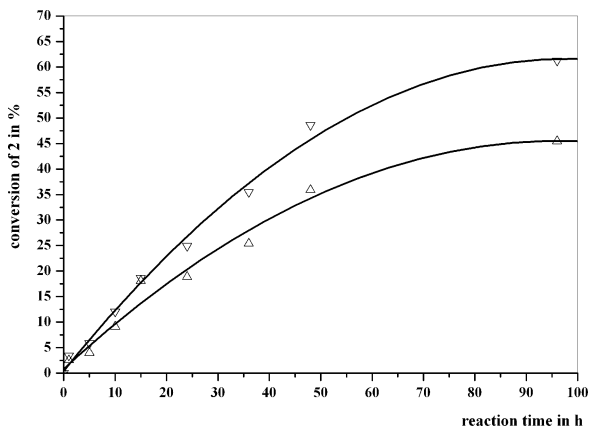

Fig. 7. Conversion of **2** by CAL B in a STR process (△) and with continuous formic acid separation in an EMR (▽).

buffer capacity had proven sufficient, and pH was not observed to change either. After 96 h, 45 % of **2** were converted, and the flattened curve indicated that chemical equilibrium was approached. A further increase in conversion could not be reached. Moreover, residual enzyme activity was 32 % after 96 h as a consequence of protein denaturation through shear forces at the membrane surfaces owing to the short distance of 1 mm between membrane and stirrer. On the other hand, since formate was continuously removed from the reaction mixture, inhibition by the latter was not responsible.

Similar effects were observed for PLE with 46 % conversion in the STR and 57 % in the EMR.

After all, another observation was drawing our attention: As can be seen from Table 2 and Fig. 7, CAL B performed poorly in the STR, but quite well in the EMR. This is the outcome of an interfacial activation through a far more effective emulsification. Yet another effect contributes to the reaction result: substrate excess inhibition. Above a siloxane **2** concentration of 30 mM, the reaction rate was observed to decrease due to half and/or fully deprotected molecules competing for entering the active site. This effect was not studied in detail since a third option came into play on which further activities were focussed.

Micro reactor

After formoxypropyl siloxane conversion to the corresponding carbinol had been shown in the EMR experiments to profit from emulsion formation between water and siloxane, options were of interest how to increase the phase interface along which formic ester hydrolysis apparently takes place. In order to check for the validity of this assumption a multilaminar micro mixer experiment was conducted. This apparatus divides the incoming fluids, water and siloxane, into alternating tiny layers, each 25 μm thick (Fig. 8).

Because in the micro-dimension diffusion is the dominant mass transfer momentum, the phases were expected to intensively mix with each other without facing immiscibility effects as observed through convective mixing. In fact, stable emulsions were formed which allowed for effective enzymatic hydrolysis of the siloxane in the loop installed downstream. A comparison between the conversions obtained in the different reactor types is given in Fig. 9.

With 61 % conversion after 96 h, the EMR had been shown to outmatch the STR in which only 45 % were obtained. One might reason that this effect is to be attributed to formic acid removal, but in fact the EMR

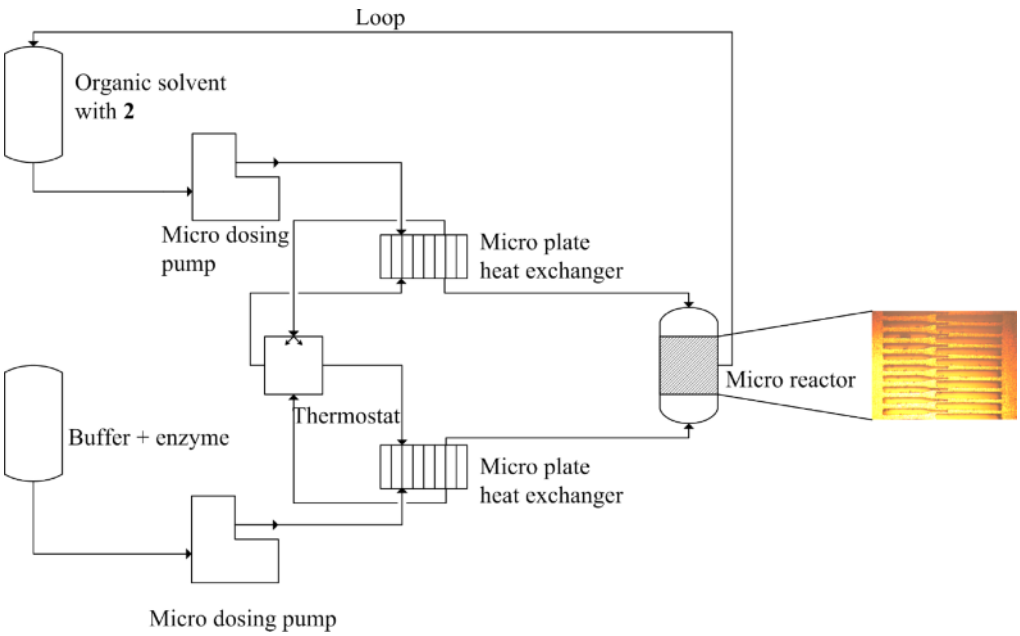

Fig. 8. Schematic setup of the micro mixer experiment.

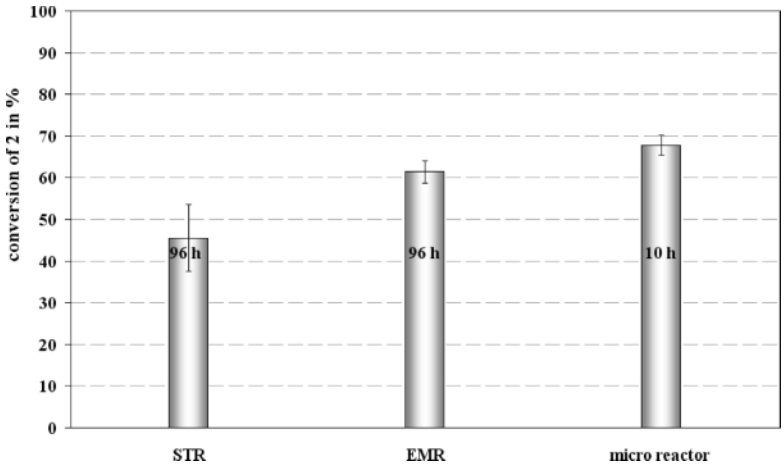

Fig. 9. Comparison of conversion of **2** by CAL B in a STR, EMR and micro reactor.

experiments were not suitable to clearly differentiate and weight the contributions of product removal and interface increase through emulsion formation. However, the impact of emulsification became obvious in the micro mixer experiments where 33 % conversion were achieved after only 60 min. By contrast, the STR required 35 h and the EMR 32 h to reach the same value. These different turnover rates support the postulate according to which a large phase interface strongly promotes the hydrolysis reaction. Through rapid diffusion-controlled siloxane transport to the interface on the one hand and rapid removal of dihydroxyalkyl siloxane and formate from the interface on the other, it becomes conceivable that these two effects efficaciously promote deprotection of **2** and shift the equilibrium to the product.

Due to the expansion of the phase interface there was also a dilution effect, which in turn reduced the effective concentration of formate as a cleavage product. At the same time the larger interface favored diffusion-controlled transport – and particularly alignment – of the space-occupying siloxane substrate. An expanded phase interface increased the proportion of substrate, but also the concentration of enzyme molecules. Thus, the reaction rate was independent of substrate concentration and solely determined by the concentration of unbound enzyme. Similar results emerged for oligomeric and polymeric formoxyalkyl siloxanes the detailed description of this phenomenon being beyond the scope of this contribution. For these species conversion ranged between 12 % – 38 % in the STR compared to 20 % – 43 % in the micro reactor.

Siloxane conversion reached 61 % within 5 h. This is the same value as obtained in the EMR, where, however, 96 h were required. Obviously the reaction equilibrium was established at that time, and emulsification was efficacious in a way that back-diffusion of formic acid to the interface and re-esterification are significantly contributing to the overall reaction under these conditions. Since no product was removed in these experiments, it is clear that the product concentration of $\alpha,\omega-$bis(3-hydroxypropyl)siloxane could not be further increased with this setup (Fig. 9).

The finding that back-diffusion of formic acid to the interface, its residence there and its re-esterification play a substantial role in the biocatalytic hydrolysis of **2** prompted us to further discuss a boundary layer rather than an interface.

Boundary layer processes in the course of the biocatalytic functionalization of organosiloxanes

The functionalization of the protected formoxyalkyl siloxane in a two-phase system results in the formation of a phase boundary layer within which a modification of the protected entity takes place. Fig. 10 illustrates the reaction **2** → **1** in simplified form and the variety of transport processes and reaction options at and within the boundary layer. The enzyme CAL B is dissolved in the aqueous phase and is activated at the boundary layer through hydrophobic **2** (E*) [12]. At the siloxane interface of the boundary layer the siloxane ester group must find access to the active site of the enzyme. This equilibrium reaction is followed by the formation of a *quasi* Michaelis-Menten complex (ES) within the

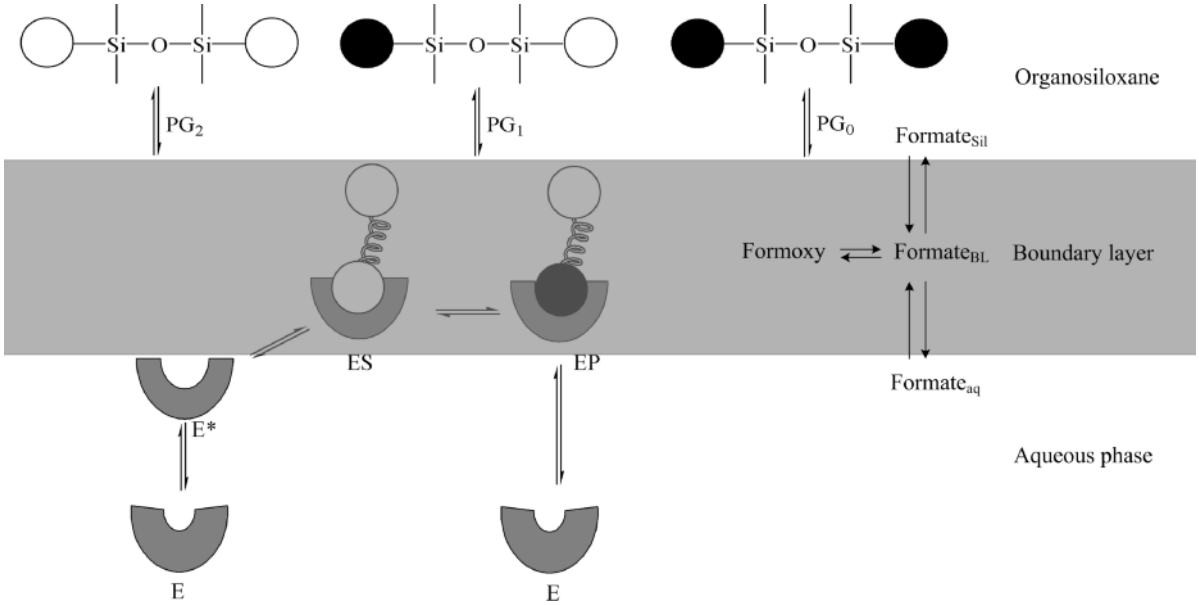

Fig. 10. Mass transfer processes in the two-phase system of organosiloxane **2** and water containing the hydrolase. The siloxane functional groups are represented by circles. PG_2 – fully protected **2**; PG_1 – half deprotected **2**; PG_0 – fully deprotected product **2**.

boundary layer. Because of the coupled phenomena of enzyme and reactant diffusion to and from the interfacial area, establishment of the phase boundary layer itself, but also the alignment of the catalytic centers towards the substrate, the kinetics of hydrolase-mediated ester cleavage inevitably does not obey exactly classical Michaelis-Menten rules. The subsequent catalytic hydrolysis leads to the release of formate, which passes from the boundary layer into the aqueous phase or from there by re-diffusion. Due to these mass transport processes the enzyme per unit of time less often docks with the actual starting material, resulting in a *de facto* inhibition of the enzyme-catalyzed process, and consequently lower turnover rates are observed. In parallel, mass transfer limitation at the junction of the boundary layer to the aqueous phase causes formate resting in the boundary layer, thus provoking a back reaction to siloxane formate **2**. Complete conversion is therefore ruled out.

The complexity of the processes in the boundary layer is increased further by the circumstance that the siloxane substrate has to dock twice with the enzyme to be fully deprotected. At the same time, half deprotected siloxane is at risk, with any approach to the hydrolase and thus to the phase boundary, to be esterified in a reverse reaction. Further investigations aimed therefore at avoiding re-esterification after mass transfer limitations between boundary layer and aqueous phase had been overcome.

The last percents to complete conversion

As pointed out before, the deprotection of **2** constitutes a major challenge since the deformylation is impeded by low substrate concentrations, phase interfacial phenomena, poor substrate solubilities as well as low functionalization densities. For the reason of the observed re-esterification of **1** within the boundary layer, and having in mind that not the phase interface itself, but the boundary layer is the actual locus where the enzymatic reaction takes place, there was the idea to render the hydrolysis irreversible by decomposing formate. An elegant approach to this aim could be the addition of formate dehydrogenase (FDH, EC1.2.1.2) together with acetone/alcohol dehydrogenase as a cofactor-regenerating system. This experimental approach raised the question whether ester cleavage would not be accomplishable by FDH directly, *e. g.* without prior hydrolase-mediated release of the formate group. FDH is well-established as a re-

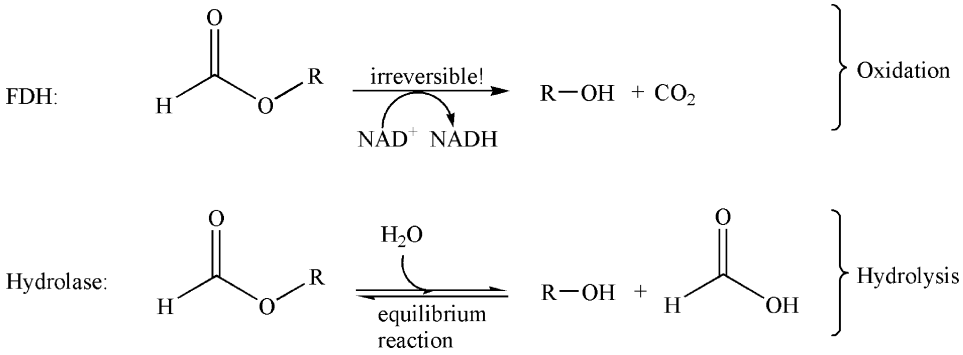

Fig. 11. Formic acid ester cleavage by FDH is an oxidation reaction while hydrolases mediate true hydrolysis.

dox enzyme for the regeneration of NADH in cofactor-dependent biocatalysis [13, 14] making use of the oxidation product CO_2 being chemically inert, while the equilibrium is completely shifted to the product through release of the gas [15]. The analogy of forming a gaseous product by treating **1** with FDH and thus irreversibly driving the reaction to full conversion prompted us to investigate FDH as a potential candidate for the cleavage of formic acid ester **1** as an oxidative alternative to classical enzyme-driven ester hydrolysis.

The catalytic mechanism of hydride transfer from formate to NAD^+ has been well examined in many theoretical and practical investigations [16 – 18]. The idea behind this concept was that if hydride abstraction was realizable with neutral, protonated formic acid, there would be no reason why the enzyme should not be capable of cleaving also formic acid esters. This oxidation reaction would yield CO_2 and the respective alcohol. It would unite conventional hydrolase-mediated ester cleavage and FDH-mediated oxidation of formate in one go (Fig. 11).

After initial attempts with simple formic acid esters, *viz.* methyl, ethyl and propyl ester, had proven successful, we tested this variant also for siloxane **2**. Advantageously, potential contributions of hydrolase activity, in concert with FDH-mediated oxidation of intermediately released formic acid, were shown to not interfere with the FDH-mediated deformylation of **2** [19]. Likewise a potential backward reaction through oxidation of isopropanol formed in the course of cofactor regeneration had no effect on the reaction, since the FDH-mediated hydrogenation of CO_2 to formic acid firstly is too slow within the time scale of this reaction, and secondly it requires pressure > 5 MPa to produce detectable amounts within 10 h.

In fact the concept held true, yet complete conversion was achieved only through a combination of micro-mixing and FDH-mediated ester cleavage. Only this fruitful interplay between two innovative strategies allowed for quantitatively releasing **1** from **2**. This process which did not require more than 10 h to be complete constitutes a quantum leap in converting water immiscible substrates in view of no more than 45 % conversion after 96 h following conventional routes.

The mechanism proposed for the novel enzymatic pathway appears to be reasonable, and the FDH process was found to be applicable to every formate ester tested so far.

Therefore, from the viewpoint of a technical application it can be concluded that for the production of terminally functionalized α,ω-hydroxyalkyl polysiloxanes the use of formylated hydroxyalkyl entities in concert with FDH-mediated deformylation in a micro mixer is an economic way and a powerful synthetic route.

Summary and Outlook

This example shows impressively the mass transfer limitations of an enzyme-catalyzed modification of formoxyalkyl siloxanes. Reaction kinetics are determined by transport processes at and within, as well as the diffusion away from the boundary layer. Different strategies, such as the use of solubilizing agents, surfactant addition and increased stirring rate led to an only moderately increased conversion, if not to impairment of enzyme activity. A successful method is the shift of the equilibrium in favor of the hydrolysis products with removal of the cleavage product formate. Through the targeted use of a multilaminar diffusion mixer, the deprotection of the form-

oxyalkyl siloxane proceeds with complete conversion at shorter reaction times than in comparable batch approaches. One strength of this approach lies in overcoming conversion-limiting interfacial effects, another one in the continuous process design. The use of a micro mixer and an enzyme system for the oxidative removal of the hydrolysis products have led to a reaction delivering the product quantitatively after 10 h, while in a stirred tank reactor 45 % conversion were not exceeded even after 96 h. Thus, the micro-process technology is well suited for two-phase systems to achieve high yields under mild conditions, rendering this approach particularly attractive for biocatalytic processes with water-immiscible compounds and thus representing a textbook example of process intensification in white biotechnology.

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