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# Activation of mandelate racemase via immobilization in lyotropic liquid crystals for biocatalysis in organic solvents: application and modeling

Christoph Bauer [a,*], Matthias Boy [b], Kurt Faber [c], Ulfried Felfer [d], Harald Voss [e]

[a] *Institut für Thermische Verfahrens- und Umwelttechnik, University of Technology, Inffeldgasse 25, A-8010 Graz, Austria*
[b] *BASF AG, ZHV/V-A015, D-67056 Ludwigshafen, Germany*
[c] *Department of Chemistry, Organic and Bio-Organic Chemistry, University of Graz, Heinrichstrasse 28, A-8010 Graz, Austria*
[d] *DSM Fine Chemicals Austria GmbH, R&D, P.O. Box 933, A-4021 Linz, Austria*
[e] *Institute of Biotechnology, University of Technology, Petersgasse 12, A-8010 Graz, Austria*

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**Abstract**

Mandelate racemase from *Pseudomonas putida* ATCC 12633 could be activated in an organic solvent by immobilization in lyotropic liquid crystals (LC). A theoretical model for a system, that consisted of an organic solvent phase to solve the nonpolar substrate and the LC phase with the immobilized enzyme was developed. The model included the biocatalytic reaction and the mass transfer in-between and within the phases. By means of the finite element method (FEM) this model was transferred into a computer program, which was used as a tool for the simulation of the racemization of D-mandelic acid by mandelate racemase in the organic solvent (di-*n*-butyl ether). The experimental results at temperatures ranging from 20 to 60°C and starting concentrations of D-mandelic acid between 40 and 80 mmol $l^{-1}$ in the organic solvent phase could be simulated by the model with high accuracy. Furthermore, the model was used to vary the geometry of the system to minimize mass transfer limitations, which represent a crucial limitation for this system. 

*Keywords:* Lyotropic liquid crystals; Enzymatic racemization; Mandelate racemase [EC 5.1.2.2]; Finite element method

## 1. Introduction

The use of organic solvents as reaction media for bioconversions has several advantages [1]: (i) increased concentrations of poorly water-soluble substrates and/or products are possible; (ii) reaction equilibria may be shifted favorably; (iii) undesired side-reactions via hydrolysis are suppressed; and many more. However, many enzymes are either inactive or are denaturated in these media. One possibility to overcome this limitation is the "activation" by the introduction of surfactants into an aqueous-organic two-phase system, which leads to the formation of reversed micelles [2]. The latter represent (often globular) microstructures of spatially oriented surfactant molecules coating a water pool within nonpolar organic solvent. Thus, enzymes can be solubilized in the water pool without loss of activity, whereas substrate/product are located in the organic phase. However, from a preparative standpoint, product recovery from reverse micelles is often difficult due to the presence of a large amount of detergent.

* Corresponding author. Tel.: +43-316-873-7984; fax: +43-316-873-7472.
*E-mail address:* bauer@tvtut.tu-graz.ac.at (C. Bauer).

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One way of avoiding this drawback of conventional reverse micellar systems is the immobilization of enzymes (or whole cells) in lyotropic liquid crystals (LC). On the one hand, immobilization facilitates the recovery of product and biocatalyst and thus allows continuous processes. On the other hand, the LC matrix, which is solid in contrast to micelles, has protective effects on the enzymes.

Lyotropic LC are found in almost every living organism [3]. They are major components of biological membranes which provide a matrix for membrane-associated proteins. These LC are surfactant aggregates and can be formed by dissolving certain amphilic molecules in organic solvent/water mixtures. Three classes of LC phase structures are known, which are arranged in lamellar, hexagonal and cubic structures. Cubic and hexagonal phases can have normal or reverse molecule orientation. For bioconversions, a two-phase system consisting of a solid LC phase (containing the immobilized enzyme) and an organic solvent phase can be used. Because of the thermodynamic equilibrium between these two phases it is possible to work with any ratio of organic solvent versus the LC phase. Poorly water-soluble substrates can be dissolved in the organic phase and products can be extracted from the LC phase.

To date, there have been only few reports on the application of LC-systems for bioconversions [4–6]. In the only previous study on the modeling of organic solvent/LC-systems [5], substrates were added directly to the stock solutions prior to the formation of the LC phase to minimize diffusional effects during the reaction and therefore, mass transfer was neglected in the model.

In extension of the previous model, an improved version for a system consisting of an organic and a liquid–crystal phase was developed in this study. It includes the enzymatic reaction by the immobilized biocatalyst as well as the coupled diffusive mass transfer of substrate and product within the solid LC phase. Differential equations with the boundary conditions were solved by the finite element method (FEM), which was initially developed by engineers to study mechanical stress problems, but has nowadays been adapted and widely used in various disciplines [7]. In this method, space is divided into a collection of small (“finite”) elements, which leads to a large system of linear equations, that can be implemented in a computer program.

The model was employed to describe a novel bioconversion in an organic solvent, i.e. the racemization of mandelic acid in di-*n*-butyl ether catalyzed by mandelate racemase [EC 5.1.2.2] from *Pseudomonas putida* ATCC 12633. Racemization is defined as formation of a racemate from a non-equimolar mixture of two enantiomers [8]. Enzymatic racemization of stereochemically stable compounds under mild conditions is of crucial importance for the conversion of a racemate into a single stereoisomer via so-called “deracemization-processes”, such as dynamic resolution [9–11]. In contrast to classic kinetic resolution, which is limited to a maximum of 50% yield of each enantiomer, the latter technique shows a significantly enhanced economic balance as it furnishes a single isomer in quantitative yield. Since for dynamic resolutions, the racemization and the enantio-selective transformation have to be performed in the same vessel, they must be compatible to each other. As a consequence, racemization by chemical means (which requires harsh reaction conditions, such as extreme pH and/or elevated temperature) is impeded, and much attention has been recently paid to the use of racemases. In this context, we have recently shown that (±)-mandelic acid can be deracemized via a two-enzyme system consisting of (i) a lipase-catalyzed acyl transfer reaction coupled to (ii) enzymatic racemization of the non-reacting substrate enantiomer by mandelate racemase at ambient temperature [12]. The latter process, however, could not be turned into a dynamic resolution due to the fact that mandelate racemase was completely inactive in a large variety of organic solvents [13]. As a consequence, the process had to be performed in a step-wise fashion going in hand with a switch of solvents from aqueous to organic. After four cycles, (*S*)-*O*-acetylmandelic acid was obtained in >80% isolated yield in >98% e.e. from (±)-mandelatic acid [12]. On an industrial-scale process, a dynamic process via combination of both biocatalyzed reactions in an organic solvent would be clearly preferable for the ease of handling.

After a lot of experimentation, including various techniques, such as enzyme modification, addition of enzyme-stabilizing and activity-modulating agents, we found that mandelate racemase from *P. putida* could be activated in di-*n*-butyl ether by

immobilization in lyotropic LC consisting of aqueous buffer, di-*n*-butyl ether and a nonionic surfactant. In order to enhance our understanding of this system, the racemization of D-mandelic acid in di-*n*-butyl ether was used for the verification of our model.

## 2. Experimental

### 2.1. Materials

(±)-, D- and L-mandelic acid and potassium hydroxide were purchased from Fluka (Buchs) and were of analytical grade. Brij® 35 (polyoxyethylene-[*n*]-dodecyl ether, $n \sim 23$), di-*n*-butyl ether, diethylether, potassium phosphate and disodium phosphate dihydrate with a purity of >99% were obtained from Merck (Darmstadt). *N*-methyl-*N*-nitroso-urea was supplied by Sigma (St. Louis). Mandelate racemase was prepared by fermentation of *P. putida* ATCC 12633 on glucose and (±)-mandelic acid based on [14]; two partially purified enzyme preparations showing a specific activity of 104 and 285 U mg$^{-1}$ (towards D-mandelic acid at 20°C and pH 7.5) were prepared in cooperation with H. Boehling.

### 2.2. Preparation of liquid crystal

Di-*n*-butyl ether (400 ml) was mixed with 20 g of the nonionic surfactant Brij® 35 and 28 ml aqueous buffer (pH 7.0; 11 g $KH_2PO_4$ and 8.9 g $Na_2HPO_4{\cdot}2H_2O$ in 1 l distilled water). This mixture was placed in a water bath (60°C) for 30 min manually shaken after 10 and 20 min to establish a two-phase system consisting of a liquid crystal- and an organic solvent phase. After cooling to room temperature, the liquid crystal was stored in the solvent phase for further use. According to additional simultaneous small and wide-angle X-ray scattering (SWAX) measurements, this liquid crystal has cubic structure [6].

### 2.3. Mass transfer experiments

Two-phase systems with a planar interphase consisting of LC- and organic solvent phase were prepared in thermostated gas-tight glass vials. First, 1 ml of liquid crystal ($\rho = 0.955$ g ml$^{-1}$) was filled into the vial with a syringe, then air bubbles were removed by a spatula and the interphase (1.04 cm$^2$) was made plane. The experiment was started by addition of aliqots of 2.5 ml di-*n*-butylether containing varying concentrations of (±)-mandelic acid. Since the chirality has no effect on diffusive mass transfer, racemic mandelic acid could be used instead of enantiopure material. Diffusion experiments were performed with pure liquid crystal as well as with liquid crystal containing 20, 40 and 60 mg of immobilized racemase per gram of LC. Racemase was immobilized by mixing the solid enzyme preparation with the liquid crystal using a Heidolph DIAX 600 high speed mixer. Starting concentrations of (±)-mandelic acid in the organic solvent phase were 20, 40, 60 and 80 mmol l$^{-1}$. The experiments were carried out at temperatures of 6, 20, 30, 40 and 60°C. Samples of 20 μl were taken from the organic solvent phase and analyzed by gas chromatography (GC) until the concentration of mandelic acid in the organic solvent phase remained constant.

### 2.4. Racemization experiments

Racemization experiments were performed in a thermostated gas-tight batch reactor shown in Fig. 1. Racemase with a specific activity of 285 U mg$^{-1}$ was immobilized by mixing the solid enzyme preparation with the liquid crystal using the high speed mixer (40 mg per gram of LC). Then, the liquid crystal phase was filled into the cylindrical cavity at the bottom of the reactor (volume 33 ml, interphase area towards

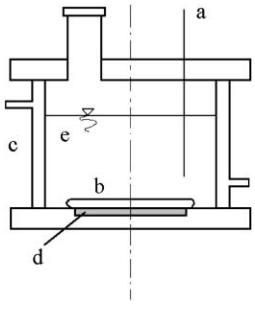


Fig. 1. Schematic illustration of the device used for racemization experiments: (a) syringe for sampling; (b) magnetic stirrer; (c) double-walled casing for circulating tempering fluid; (d) LC phase in cavity; (e) organic solvent phase.

the organic solvent phase: 30.2 $cm^2$). To avoid the formation of a liquid side film, a magnetic stirrer was mounted directly over the liquid crystal phase. Then, 250 ml of di-*n*-butyl ether containing various starting concentrations of D-mandelic acid was filled into the thermostated reactor at various temperatures: $c_0 = 40\,mmol\,l^{-1}$ at $T = 20°C$; $c_0 = 40\,mmol\,l^{-1}$ at $T = 60°C$; $c_0 = 60\,mmol\,l^{-1}$ at $T = 40°C$; $c_0 = 80\,mmol\,l^{-1}$ at $T = 20°C$; $c_0 = 80\,mmol\,l^{-1}$ at $T = 60°C$. Stirring at 70 rpm was started by the control device under the reactor. Samples were withdrawn via syringe from the organic solvent phase until the product concentration in the organic solvent phase reached a maximum.

### 2.5. Sample preparation for GC-analysis

Aqueous KOH solution (1 ml, 40%) was mixed with 3 ml diethylether in an ice bath and 13.9 mg *N*-methyl-*N*-nitroso-urea was added. The mixture was stirred at 0°C for 10 min in a well ventilated compartment (caution: $CH_2N_2$) until the ether phase turned yellow. A 20 μl aliquot of each sample was diluted with 500 μl dibutylether and mixed with 200 μl yellow ether phase in GC-vials. The vials were stored at 4°C for analysis.

### 2.6. GC-analysis

GC-analysis was performed on a Hewlett-Packard 5890 Series II plus chromatograph equipped with FID and an HP 6890 auto injector. Column: Chrompack Chirasil DEX, 25 m, $N_2$ carrier. Method: initial temperature 110°C for 20 min, then at $30°C\,min^{-1}$ rate until a final temperature of 160°C was reached, hold for 3 min. Retention times of mandelic acid methyl ester: D 16.7 min; L 17.8 min.

## 3. Results and discussion

### 3.1. Mass transfer experiments

The results from the mass transfer experiments indicate a temperature dependence of the diffusion into and within the LC phase. Fig. 2 shows the concentration of mandelic acid in the organic solvent phase for the first 40 h for $80\,mmol\,l^{-1}$ starting concentration of mandelic acid at 6 and 60°C. For a given start concentration at all temperatures, the same final concentration of mandelic acid in the organic solvent phase was reached at the equilibrium point (not shown). No influence of mass transfer by

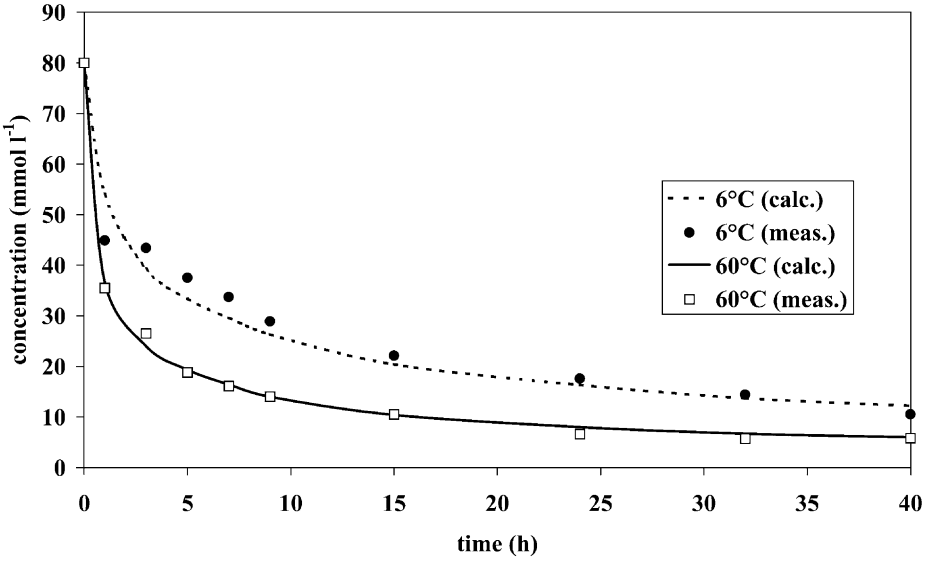


Fig. 2. Concentration of mandelic acid in the organic phase during mass transfer experiments at 6 and 60°C, start concentration $80\,mmol\,l^{-1}$ (measured and modeled).

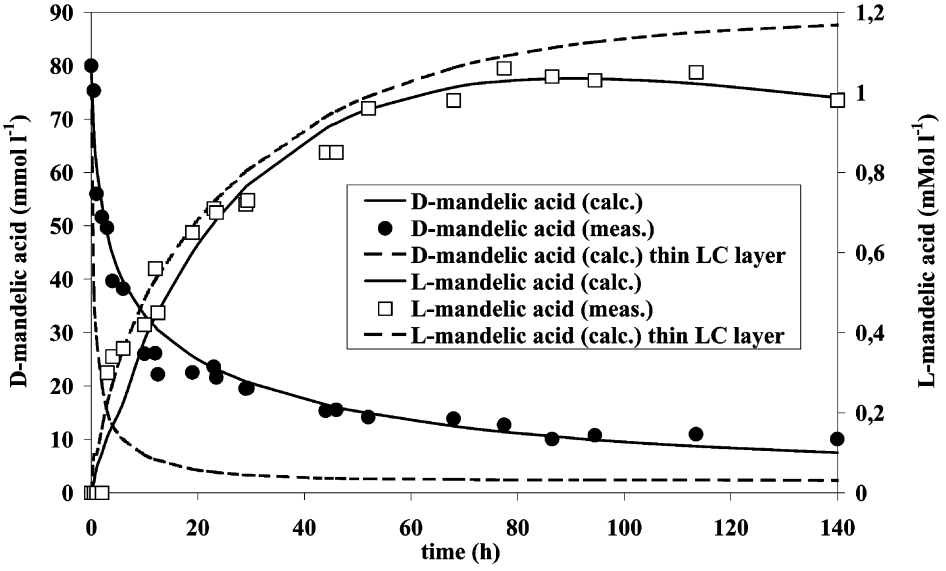


Fig. 3. Concentration of D-mandelic acid and L-mandelic acid during racemization at 60°C, start concentration 80 mmol $l^{-1}$ (measured and modeled) and calculated concentrations for a simulated experiment at the same conditions but with an interphase area of 110.5 $cm^2$.

immobilized enzyme could be observed for both enzyme preparations.

### 3.2. Racemization experiments

In all racemization experiments, the same effects were observed. At the beginning, D-mandelic acid was transported into the unloaded LC phase by diffusive mass transfer, while no L-mandelic acid could be detected in the organic solvent. After a while, the concentration of L-mandelic acid increased. The LC phase remained solid at all temperatures. One interesting effect is shown in Fig. 3 (showing substance concentrations for a start concentration of 80 mmol $l^{-1}$ D-mandelic acid in the organic solvent phase at 60°C). After reaching a maximum, a slight decrease of product concentration in the organic solvent phase was observed. The reason for this behavior will be explained later in the paper by the results of the model simulations.

### 3.3. Model

The results of the mass transfer and reaction experiments served as a basis for the verification of a model, which was developed for a two-phase system and consists of a LC phase (containing immobilized enzyme) and an organic solvent phase.

In this one-dimensional model (Fig. 4), mass transport within a homogeneous solid phase (LC) is coupled to a reaction within this phase. A liquid side film at the interphase was neglected, because of the magnetic stirrer placed directly above the LC phase.

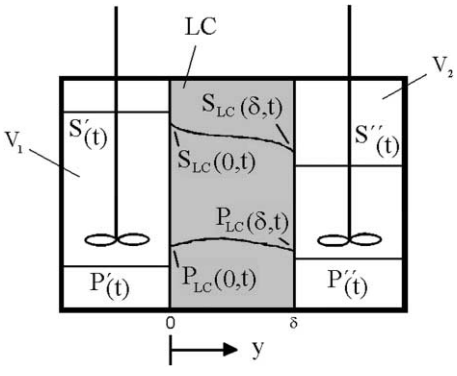


Fig. 4. Model of the multiphase system. $V_1$, $V_2$: organic solvent phases; LC: LC phase; $S'(t)$, $P'(t)$, $S''(t)$, $P''(t)$: concentrations of substrate and product in the organic solvent phases; $S_{LC}(y,t)$, $P_{LC}(y,t)$: concentrations of substrate and product in the LC phase; $y$: gradient in LC phase.

Table 1
Mathematical model of a multiphase system consisting of organic solvent and liquid crystal

Diffusive mass transfer and reaction in the solid LC phase

(1) $\dfrac{\partial S_{LC}(y,t)}{\partial t} = D_S \dfrac{\partial^2 S_{LC}(y,t)}{\partial y^2} - r$

(2) $\dfrac{\partial P_{LC}(y,t)}{\partial t} = D_P \dfrac{\partial^2 P_{LC}(y,t)}{\partial y^2} + r$

(3) $r = \dfrac{\partial P_{LC}}{\partial t} = \left[ \dfrac{r_{max} S_{LC}}{S_{LC} + K_M} - \dfrac{r_{max} P_{LC}}{P_{LC} + K_M} \right] \exp(-K_D t)$

$S_{LC}$, $P_{LC}$: concentrations of substrate S and product P in LC phase (mol $l^{-1}$); $D_S$, $D_P$: diffusion coefficients of substrate S and product P in LC phase ($cm^2$ $s^{-1}$); $r$: reaction rate in LC phase (mol $l^{-1}$ $s^{-1}$); $t$: time (s); $y$: position in LC (cm); $K_M$: Michaelis–Menten constant (mol $l^{-1}$); $K_D$: deactivation parameter ($s^{-1}$); $r_{max}$: maximum velocity (mol $l^{-1}$ $s^{-1}$)

Boundary conditions of the system

(4) $S_{LC} = 0, \quad P_{LC} = 0, \quad t = 0$
(5) $S_{LC}(0,t) = K_S S'(t)^{ns}, \quad y = 0, \quad t > 0$
(6) $P_{LC}(0,t) = K_P P'(t)^{np}, \quad y = 0, \quad t > 0$
(7) $S_{LC}(\delta,t) = K_S S''(t)^{ns}, \quad y = \delta, \quad t > 0$
(8) $P_{LC}(\delta,t) = K_P P''(t)^{np}, \quad y = \delta, \quad t > 0$

$K_S$, $K_P$: distribution coefficients of substrate S and product P; ns, np: Freundlich-exponents of substrate S and product P; $P'$, $S'$: product and substrate concentrations in organic solvent phase 1 (mol $l^{-1}$); $P''$, $S''$: product and substrate concentrations in organic solvent phase 2 (mol $l^{-1}$)

Mass balances for organic solvent phase $V_1$ and $V_2$ for substrate and product

(9) $\dfrac{d(S'V_1)}{dt} = -F_{Interphase} D_S \dfrac{dS_{LC}(y=0,t)}{dy}$

(10) $\dfrac{d(P'V_1)}{dt} = -F_{Interphase} D_P \dfrac{dP_{LC}(y=0,t)}{dy}$

(11) $\dfrac{d(S''V_2)}{dt} = -F_{Interphase} D_S \dfrac{dS_{LC}(y=\delta,t)}{dy}$

(12) $\dfrac{d(P''V_2)}{dt} = -F_{Interphase} D_P \dfrac{dP_{LC}(y=\delta,t)}{dy}$

$F_{Interphase}$: interphase between organic solvent phase and LC phase ($cm^2$); $V_1$, $V_2$: organic solvent phases ($cm^3$)

In order to obtain a more general model, a liquid bulk phase (representing the organic solvent) was implemented at both sides of the LC phase. This could be helpful in industrial processes, where one liquid phase can be used to transport the substrate into the LC phase while the second extracts the product from the LC.

This model could be mathematically described by the equations presented in Table 1. The concentrations of substrate and product in the liquid organic bulk phase serve as boundary conditions, while the Freundlich adsorption law was used for the distribution of substrate and product at the interphase between organic solvent and LC. The reaction kinetics consist of a Michaelis–Menten-type in both directions with the same kinetic parameters. Such an approach for biocatalytic racemization was verified by several studies [15–17]. Time-dependent enzyme deactivation during the reaction was considered by a deactivation term. Further experiments indicate (data not shown), that deactivation was of first-order.

The mathematical system of differential equations could be solved only by numerical techniques, therefore the FEM was used for this study. Based on modules presented in [18], a computer program was written using the software package MATLAB 4.0 running on a Pentium 133 MHz PC with Windows 95 and the values for the model parameters were determined.

First, solely mass transfer in the absence of racemization was examined. For the calculation, the LC phase was divided into 18 layers and only one solvent

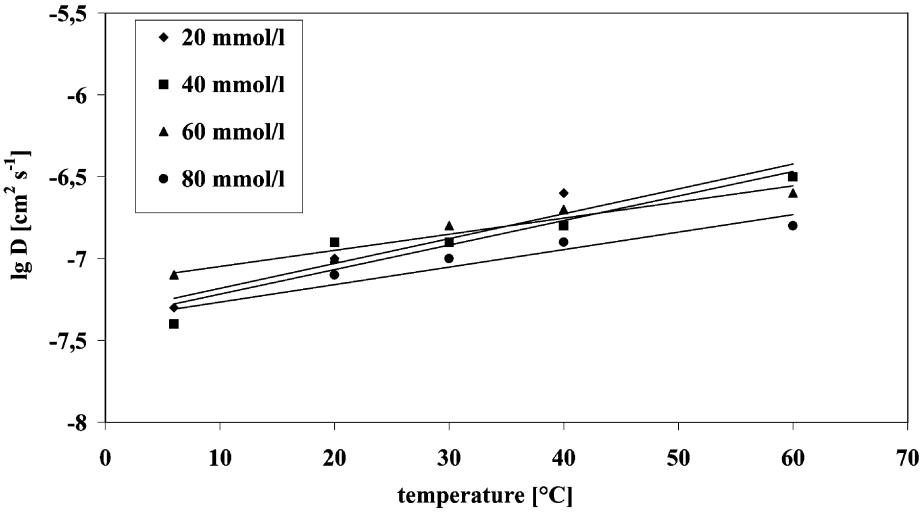


Fig. 5. Diffusion coefficients modeled for mass transfer experiments at different start concentrations.

phase was used. The results describing the diffusion coefficients are presented in Fig. 5. A linear increase of the logarithm plot of the diffusion coefficients with increasing temperature indicate an Arrhenius law temperature dependency. The distribution coefficients were nearly constant ($K \approx 34$) for all starting concentrations and temperatures. The value for the Freundlich exponent ($n$) was always equal 0.75. These constant values for the parameters $K$ and $n$ fit well to the effect of the same concentrations at equilibrium in the mass transfer experiments for all temperatures for a given start concentration. A comparison between experimentally determined and calculated concentrations is shown for experiments with 80 mmol $l^{-1}$ starting concentration of mandelic acid in the organic solvent phase at 6 and 80°C in Fig. 2, indicating a good correlation.

Based on the results from mass transfer experiments, the racemization experiments were simulated. The results for $r_{max}$, $K_M$ and the deactivation parameter $K_D$ are shown in Table 2. The parameters $r_{max}$ and $K_D$ increased several fold with increasing temperature, while $K_M$ remained constant. The $K_M$ value of 0.015 mol $l^{-1}$ correlates well with the $K_M$ value of 0.02 mol $l^{-1}$ previously reported from aqueous media [19]. The results of the model calculations fit well with the experimental data from the racemization experiments as shown in Fig. 3 for 80 mmol $l^{-1}$ start concentration of D-mandelic acid at 60°C.

After verification of the model, it was used for further examination of the reaction system. By computer calculation, the distribution of the substances in the solid LC phase were calculated and are presented in a diagram. Thus, the distribution of D- and L-

Table 2
Results from model calculations for kinetic reaction parameters of racemization experiments[a]

| Experimental conditions | | Parameters | | |
|---|---|---|---|---|
| $c_0$ (mmol $l^{-1}$) | $T$ (°C) | $r_{max}$ (mol/($l_{LC}$ s)$^{-1}$) | $K_M$ (mol $l^{-1}$) | $K_D$ ($s^{-1}$) |
| 40 | 20 | 0.5E−5 | 0.015 | 0.17E−5 |
| 80 | 20 | 0.5E−5 | 0.015 | 0.16E−5 |
| 60 | 40 | 1.1E−5 | 0.015 | 0.25E−5 |
| 40 | 60 | 2.7E−5 | 0.015 | 0.74E−5 |
| 80 | 60 | 2.5E−5 | 0.015 | 0.70E−5 |

[a] $r_{max}$: maximum rate; $K_M$: Michaelis–Menten constant; $K_D$: deactivation parameter.

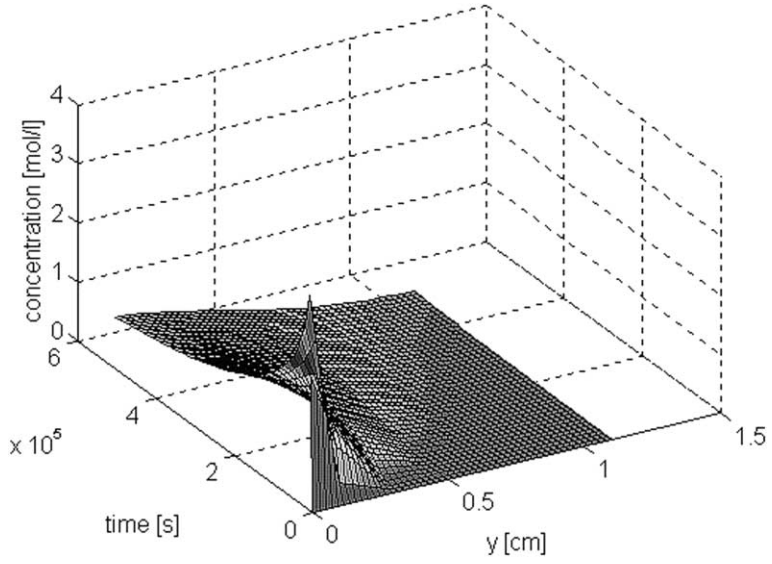


Fig. 6. Concentration of D-mandelic acid in the LC phase during racemization at 60°C, start concentration 80 mmol $l^{-1}$; *y*: gradient in LC layer with $y = 0$ at the interphase.

mandelic acid during the racemization experiment for 80 mmol $l^{-1}$ start concentration in the organic solvent phase at 60°C are shown in Figs. 6 and 7. It was observed, that under these conditions, mass transfer indeed represented a limiting factor, which resulted in high substance-concentration within the LC phase close to the interphase. As a consequence, a vast amount of immobilized enzyme was not exposed to a sufficient concentration of D-mandelic acid and thus could not participate in the reaction. These results

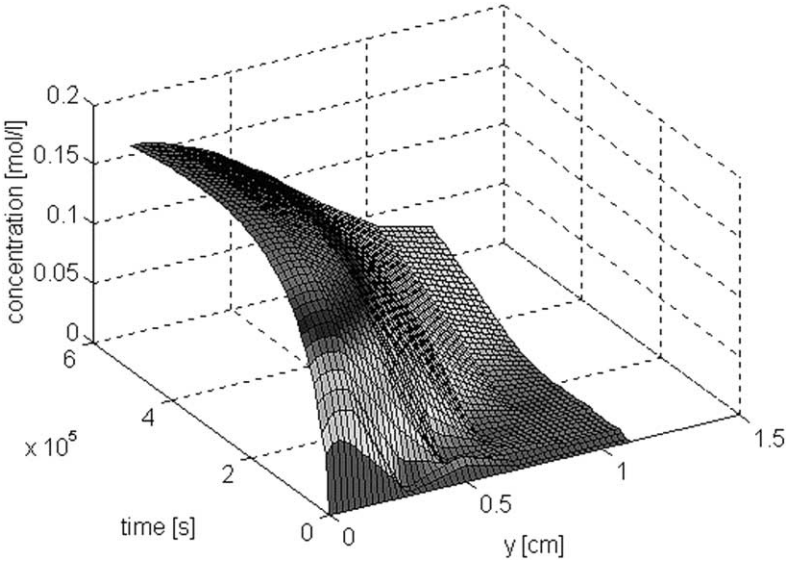


Fig. 7. Concentration of L-mandelic acid in the LC phase during racemization at 60°C, start concentration 80 mmol $l^{-1}$; *y*: gradient in LC layer with $y = 0$ at the interphase.

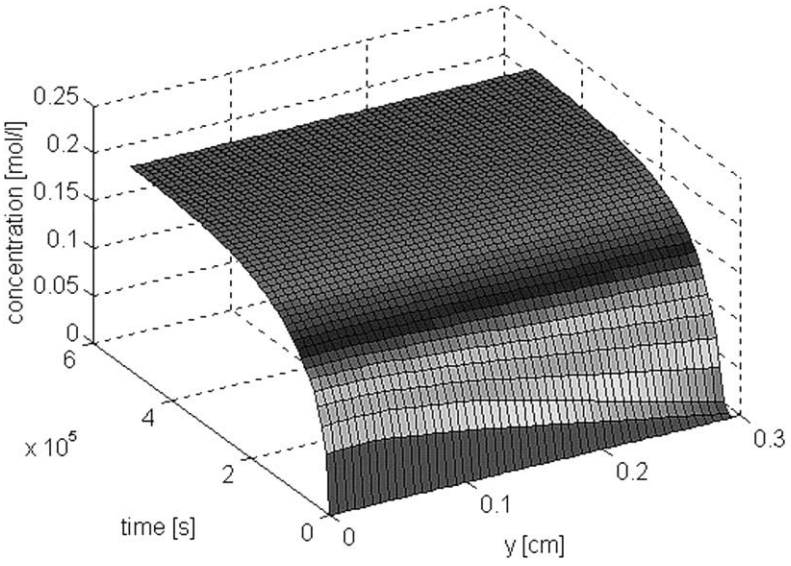


Fig. 8. Concentration of L-mandelic acid in the LC phase during simulated racemization at 60°C, start concentration 80 mmol $l^{-1}$. Interphase area: 110.5 $cm^2$; $y$: gradient in LC layer with $y = 0$ at the interphase.

also explain the effect from experiment shown above (Fig. 3), where the concentration of L-mandelic acid in the organic solvent decreased slightly after having reached a maximum. A high reaction rate at the start leaded to high concentration of L-mandelic acid in the LC area near the interphase with high mass transfer of L-mandelic acid into the organic solvent. Later the product shifts the reaction towards equilibrium which lowers the reaction rate. At the same time, L-mandelic acid is further distributed by diffusion from the LC layers of high concentration — close to the interphase — to the LC layers at the bottom of the cavity. As a consequence, the concentration of L-mandelic acid in the layers near the interphase decreases which finally results in a temporary reversion of the mass transfer at the interphase.

In technical process applications, limitations by mass transfer have to be avoided. The latter may be easily accomplished by enlarging the interphase area between the organic solvent and the LC, e.g. by employing a thin sheet of LC. Thus, conditions were modeled for a reduced LC layer thickness of only 0.3 cm (instead of 1.02 cm), resulting in an increased interphase area of 110.5 $cm^2$ for the same LC volume and amount of enzyme. The calculated results (depicted in Fig. 8) show that there is a nearly constant product-distribution in the LC phase, which indicates that the immobilized enzyme preparation was being actively used as a whole. This results in a faster increase of concentration of L-mandelic acid (Fig. 3) in the organic solvent phase and the reversion of mass transfer of L-mandelic acid at the interphase is negligible.

Good examples for technical realization of such a concept with thin LC layers might be spiral modules as known from membrane filtration. Such modules, which allows continuous processing could be described by the same mathematical model as presented in this paper, with an extension of the FEM into two dimensions.

## 4. Conclusions

The modeling of a two-phase system containing a biocatalyst immobilized in an activating LC phase and the substrate being dissolved in an organic phase was achieved. A highly unstationary case of a batch reaction could be calculated with high accuracy, where the LC phase was completely unloaded with substrate, leading to strong mass transfer limitation. The model can serve as a basis for the simulation of such a system

in a technical process, where mass transfer limitation has to be prevented. This can be achieved via an optimal design of the LC layer, which leads to an even substrate/product-distribution in the solid LC matrix. As a consequence, all of the biocatalyst can participate in the reaction leading to a maximal reaction rate.

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