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Modelling the Reaction Course of a Dynamic Kinetic Resolution of Amino Acid Derivatives: Identifying and Overcoming Bottlenecks

Vera Zimmermann,[†] Matthias Beller,[‡] and Udo Kragl*,[†]

Universität Rostock, Abteilung für Analytische, Technische und Umweltchemie, Albert-Einstein-Strasse 3a, 18059 Rostock, Germany, and Leibniz-Institut für Katalyse an der Universität Rostock e.V., Albert-Einstein-Strasse 29a, 18059 Rostock, Germany

Abstract:

Theoretically, dynamic kinetic resolution gives stereochemically pure compounds in 100% yield. Practically, this target is seldom reached. In this work, we identified limiting factors for a given reaction. The dynamic kinetic resolution of α-amino acid esters at 25 °C in a water/acetonitrile mixture gives optically active α-amino acids in good yields and optical purity. The Alcalase-catalysed hydrolysis of the ester is combined with in situ racemisation catalysed by 3,5-dinitrosalicylaldehyde. Kinetic analysis and modelling of the system made optimisation possible. Determination of reaction kinetics showed that catalyst deactivation is the limiting factor, and modelling and optimisation of the reaction system lead to an improved yield and shorter reaction time.

1. Introduction

The synthesis of chiral, enantiomerically pure compounds still attracts growing attention in chemical synthesis due to the increasing need for such compounds in the pharmaceutical and agricultural industries.[1,2] Although asymmetric catalysis has developed quickly during the past decades, chiral compounds are often obtained via resolution of racemic mixtures.[3,4] The disadvantages of this procedure are well-known: The maximum product yield cannot exceed 50%, and the remaining substrate has to be discarded or recycled by more or less intricate racemisation. The dynamic kinetic resolution (DKR) is an improvement of the kinetic resolution and avoids these drawbacks. During DKR the kinetic resolution of a racemic mixture is coupled with in situ racemisation of the substrate so that all of the substrate can be converted into the desired, enantiomerically pure product.[5] There are several excellent reviews summarising the systems developed so far.[6−11] Theoretically, the dynamic kinetic resolution offers a tool to prepare optically active compounds with 100% yield and 100% ee.[12] Experience shows that this aim is practically seldom reached. In this work we investigated the kinetics of a known DKR and modelled the course of reaction to examine the factors that are limiting for yield and optical purity.

The reaction examined was the dynamic kinetic resolution of DL-phenylalanine ethyl ester shown in Scheme 1.[13] Several examples for the DKR of amino acid derivatives using protease-catalysed hydrolysis combined with substrate racemisation catalysed by an aldehyde have been reported,[14−17] but all of them suffer from disadvantages such as low yield or optical purity or high catalyst concentration or the use of very expensive catalysts. In our case the Alcalase-catalysed hydrolysis of the ester is coupled with in situ racemisation catalysed by 3,5-dinitrosalicylaldehyde **5**. The major enzyme component in Alcalase is subtilisin (E.C. 3.4.21.62). The reaction is carried out at 25 °C in a 1:1 mixture of water and acetonitrile and an apparent pH of 7.5 and gives L-phenylalanine **3** in 80% yield and 96% ee after 30 h of reaction time. These were the conditions giving the best results during development of the reaction system in previous studies.[18]

To examine why the yield is limited to 80%, the kinetics of the reaction were determined, and the course of the reaction was modelled. We show in the following that a deactivation of the catalysts is the limiting factor in this reaction and how the obtained yield can be raised while shortening the reaction time.

* To whom correspondence should be addressed. Telephone: +49 381/498-6450. Fax: +49 381/498-6452. E-mail: udo.kragl@uni-rostock.de.

† Universität Rostock.

‡ Leibniz-Institut für Katalyse an der Universität Rostock.

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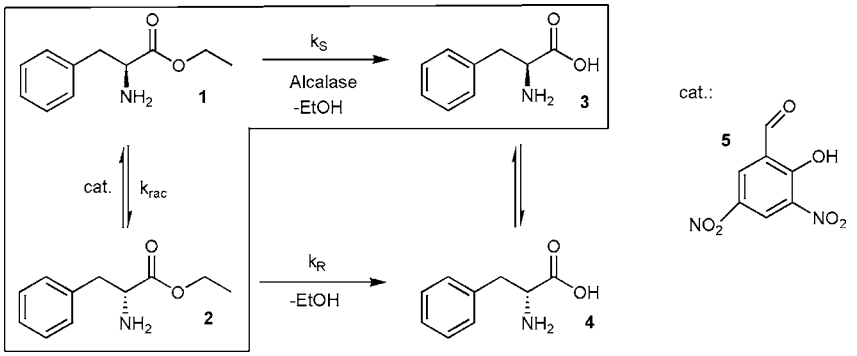
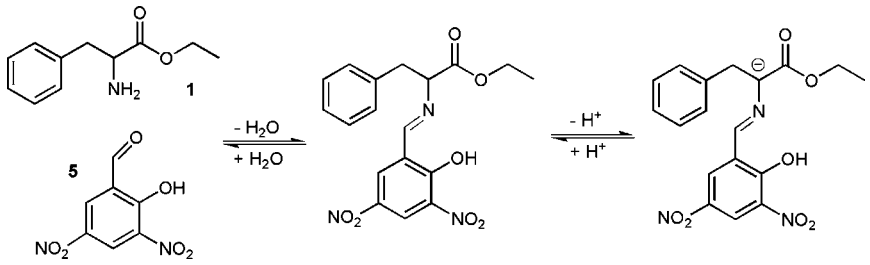
Scheme 1. **Dynamic kinetic resolution of DL-phenylalanine ethyl ester**[a]



[a] Desired reaction (inside frame) and side reactions.

Scheme 2. **Mechanism of the racemisation of phenylalanine ethyl ester**



2. Experimental Section

Chemicals. L-Phenylalanine and L-phenylalanine ethyl ester were obtained from Fluka (Buchs, Switzerland). L-phenylalanine ethyl ester was always applied as the hydrochloride. 3,5-Dinitrosalicylaldehyde and perchloric acid were obtained from Sigma-Aldrich (Steinheim, Germany). Alcalase was purchased as a brown liquid from Merck (Darmstadt, Germany) with a specific activity of 0.6 Anson-U/g. Sodium hydroxide was obtained from Lachema (Prague, Czech Republic), acetonitrile and methanol from J. T. Baker (Deventer, Netherlands).

Kinetic Constants. The kinetic constants were obtained by determination of initial reaction rates for various substrate concentrations at a constant temperature of 25 °C in a solvent mixture of acetonitrile and water (1:1). Substrate concentrations were varied between 0.03 and 1.2 mol/L while the catalyst concentrations remained constant (0.005 mol/L dinitrosalicylaldehyde or 1 vol % Alcalase). Periodically, aliquots of 100 μL were withdrawn and diluted 1:10 in hydrochloric acid (pH 1). The solution was filtered with MICROCON *YM* 30 centrifugal filters and analysed by HPLC.

DKR. DL-Phenylalanine ethyl ester was dissolved in 1:1 mixture of water/acetonitrile to give a 0.2 mol/L solution. 3,5-Dinitrosalicylaldehyde was dissolved in this solution to give a concentration of 0.005 mol/L. The pH of the solution was adjusted to 7.5 with concentrated sodium hydroxide and controlled during the reaction. To start the reaction, 1 vol % of Alcalase was added. Aliquots were treated as described above. The reaction was carried out at 25 °C.

HPLC. The concentrations of D- and L-phenylalanine ethyl ester and D- and L-phenylalanine were determined by HPLC-measurement using a DAICEL Crownpak CR(−) analytical column. Samples were eluted at 10 °C with a mixture of perchloric acid (pH = 2) and methanol 9:1 at 1.5 mL/min, and monitored at 265 nm.

3. Results and Discussion

Kinetic Model. To model the reaction course of the dynamic kinetic resolution, the kinetics of every reaction step were determined. The reactions taking place simultaneously during the dynamic kinetic resolution can be distinguished in Scheme 1. The enzymatic hydrolysis of L-phenylalanine ethyl ester **1** is coupled with the aldehyde-catalysed racemisation of the substrate. Possible side reactions are the cleavage of D-phenylalanine ethyl ester **2** by the enzyme, racemisation of the product L-phenylalanine and the base-catalyzed chemical saponification of both D- and L-phenylalanine ethyl ester. The reaction kinetics for the main reaction steps were determined by initial rate measurements.

Racemisation. It has formerly been assumed that racemisation of amino acid esters in the presence of aldehydes takes place via Schiff base formation and is well described in the literature[19,20] (Scheme 2). The mechanism of the racemisation of amino acid derivatives via Schiff base intermediates is described by Wegman et al.[20] After formation of the Schiff base the acidic proton located at the stereogenic center is removed under alkaline conditions to form an enolate species which has lost every optical information.

The Michaelis−Menten-like saturation kinetics reported by Wegman et al.[20] for this type of racemisation could not be observed in our case. This is likely due to the different reaction conditions, substrates, and catalysts. The reaction

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kinetics reported by Wegman et al. corresponded to phenylglycine methyl ester and phenylglycine amide racemised by pyridoxal and salicylaldehyde in *tert*-butyl alcohol. For the racemisation of phenylalanine ethyl ester the initial racemisation rate was linearly proportional to the initial concentration in a concentration range from 0.03 to 0.3 mol/L. The reaction kinetics can be described by first-order kinetics according to:

$$\nu = \frac{\mathrm{d[LE]}}{\mathrm{d}t} = k'_{rac}[\mathrm{LE}]$$

with

$$k'_{\mathrm{rac}} = k_{\mathrm{rac}}[\mathrm{cat}] \qquad (1)$$

k_{rac} is replaced by k'_{rac}, corresponding to k_{rac} multiplied by the concentration of 3,5-dinitrosalicylaldehyde, because the catalyst concentration is assumed to be constant during the reaction. k'_{rac} was determined to be $(3.7 \pm 0.5) \times 10^{-3}$ min^{-1} at a catalyst concentration of 0.005 mol/L. Hence k_{rac} has a value of 0.74 min^{-1}. The kinetics found are probably still concordant with the mechanism and kinetics proposed by Wegman et al. because saturation kinetics can be approximated as first-order kinetics if the concentration range determined is below the value of the dissociation constant K_{diss} of the pre-equlibrium. This constant is probably higher than 0.3 mol/L in this case.

Enzymatic Hydrolysis. The enzymatic saponification of L-phenylalanine ethyl ester obeyed Michaelis−Menten kinetics according to:

$$\nu = \frac{V'_{\mathrm{max}}[\mathrm{LE}]}{K_{\mathrm{M}} = [\mathrm{LE}]} \qquad (2)$$

with $K_{\mathrm{M}} = 0.74 \pm 0.2$ mol/L and $V'_{\mathrm{max}} = (3.5 \pm 0.5) \times 10^{-3}$ mol/(L·min). No data for this or similar solvent systems can be found in the literature although L-phenylalanine ethyl ester is a well-known substrate for subtilisin. In aqueous ethanol (7% ethanol, 30 °C, pH 7.5) a K_{M} value of 0.48 mol/L was determined.[21] Both K_{M} values are in the same order of magnitude.

The initial substrate concentration was varied between 0.03 and 1.2 mol/L. To investigate possible product inhibition, the initial reaction rate was determined at fixed substrate concentrations (0.05, 0.07, and 0.1 mol/L) with varying amounts of product added (0−0.12 mol/L). No substrate or product inhibition was observed.

Side Reactions. Kinetic rate equations for all possible side reactions were determined as well. The investigations showed that the chemical saponification of DL-phenylalanine ethyl ester had to be taken into account, while the reaction rate of the product racemisation was negligible. The saponification was examined with L-phenylalanine ethyl ester as substrate. The reaction proceeded very slowly so that the kinetics could not be determined by initial rate measurements. The course of the reaction was observed over 3 days via the decrease of substrate concentration and could be described by first-order kinetics:

$$\nu = \frac{\mathrm{d[LE]}}{\mathrm{d}t} = k'_{\mathrm{sap}}[\mathrm{LE}]$$

with

$$k'_{\mathrm{sap}} = k_{\mathrm{sap}}[\mathrm{H_2O}] = (9.0 \pm 2.4) \times 10^{-6}\ \mathrm{min}^{-1} \qquad (3)$$

Because the water concentration is constant, k_{sap} is replaced by k'_{sap}, corresponding to k_{sap} multiplied by the water concentration. A comparison of the reaction rates shows that the enzymatic cleavage is about 350-fold faster than the chemical cleavage.

Observation of the racemisation of L-phenylalanine in the presence of 3,5-dinitrosalicylaldehyde showed very little product formation after 3 days, but the HPLC peak was to small to be analysed. Compared with the other reaction rates determined, the rate of product racemisation was too slow to be taken into account.

The knowledge of the kinetic equations for every reaction step enabled us to determine the enantiomeric ratio E, which describes the ratio of the enzymatic conversion of both substrate enantiomers,[22] and the ratio of racemisation and enzymatic cleavage velocity $k_{\mathrm{rac}}/k_{\mathrm{S}}$. The knowledge of these parameters allows an estimation of the efficiency of the DKR.[23] For an efficient dynamic kinetic resolution, the parameters E and $k_{\mathrm{rac}}/k_{\mathrm{S}}$ have to be carefully tuned.[12] To achieve good optical purity of the product (more than 95% ee), E has to be greater than 50 while the ratio of the kinetic constants k_{rac} and k_{S} should exceed at least 1 to avoid a depletion of the reacting substrate enantiomer which is, in this case, L-phenylalanine ethyl ester. The optical purity of the product depends on these parameters as shown in Figure 1.

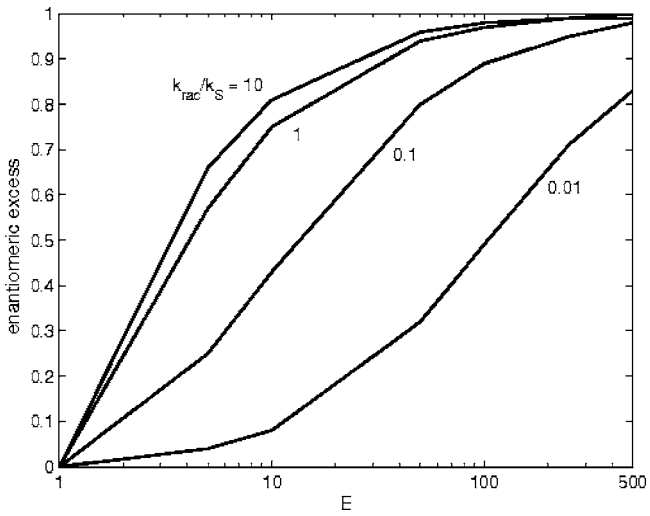

Figure 1. **Optical purity of the product depends on the enantiomeric ratio E of the enzymatic ester cleavage and the ratio k_r/k_S.**

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The E values found for the Alcalase-catalysed cleavage varied between 120 and 630. The E value was calculated from the ee values of product and substrate:[24]

$$E = \frac{\ln\dfrac{1 - \mathrm{ee_s}}{1 + (\mathrm{ee_s}/\mathrm{ee_p})}}{\ln\dfrac{1 + \mathrm{ee_s}}{1 + (\mathrm{ee_s}/\mathrm{ee_p})}}$$

This big variance is probably due to the difficult determination of ee values higher than 98%. Anyway, the observed E value is by far high enough for an efficient kinetic resolution.

For determination of k_{rac}/k_S, the Michaelis−Menten-kinetics found for the enzymatic saponification of **1** was approximated by first-order kinetics for concentrations smaller than K_M, using k_S as kinetic constant. The value found for k_S was 3.2×10^{-3} min^{-1} which resulted in a ratio $k_{rac}/k_S = 1.2$ under the chosen reaction conditions. This simplification is possible as most of the reaction has to be performed at substrate concentrations less than 0.3 mol/L due to the limited substrate solubility.

Kinetic Model. We next combined the kinetic equations for all reaction steps to model the reaction course. The system of four differential equations describing the concentration of D- and L-phenylalanine ethyl ester and D- and L-phenylalanine during reaction was solved using *MATLAB*.[25] Because the model was derived for a constant pH, reactive forms of the components and hence dissociation constants for acidic groups are not considered. The HPLC analysis applied allowed the determination of components **1**−**4** concurrently. First, experiments showed that the developed model was not able to describe the reaction course completely over 30 h. The reaction proceeded more slowly than predicted by the model and terminated at about 70% conversion. Because there was no product inhibition found, a deactivation of one or both catalysts could be an explanation. Further investigations showed that both catalysts lose activity when exposed to the reaction system (data not shown). The model was adjusted to this by introducing a new factor into the kinetic equation for racemisation as well as enzymatic hydrolysis. As the catalyst concentrations were assumed to be constant, they were included in k'_{rac} and V'_{max}. This is now used to describe the deactivation of the catalysts. Assuming first-order kinetics, the deactivation can be described as a depletion of the catalyst concentration for both catalysts:

$$k'_{rac} = k_{rac}[\mathrm{cat}]\exp(-k_{Drac}t) \quad (4)$$

$$V'_{max} = V_{max}[E]\exp(-k_{De}t) \quad (5)$$

The values for the deactivation constants k_{Drac} and k_{De} were calculated and optimised by least-squares fitting using the software *SCIENTIST*.[26] For this fitting one dataset of measured concentrations of the reaction performed under standard conditions was used. The constants are shown in Table 1. The enzyme loses about 7% of its initial activity within the first hour of reaction time, the salicylaldehyde, about 5%. With this improved model the reaction course of the DKR could be described completely as shown in Figure 2. The model has been verified using catalyst concentrations within a range from 0.005 to 0.05 mol/L and substrate concentrations within a range from 0.04 to 0.2 mol/L. The Alcalase concentration was varied from 1 to 5 vol % (data not shown).

Table 1. **Kinetic constants**

constant	value
k'_{rac}	$(3.7 \pm 0.5) \times 10^{-3}$ min^{-1}
K_M	0.74 ± 0.20 mol/L
V_{max}	$(3.5 \pm 0.5) \times 10^{-3}$ mol/(L · min)
k'_S	$(9.0 \pm 2.4) \times 10^{-6}$ min^{-1}
k_{Drac}	$(1.1 \pm 0.3) \times 10^{-3}$ min^{-1}
k_{De}	$(1.2 \pm 0.1) \times 10^{-3}$ min^{-1}

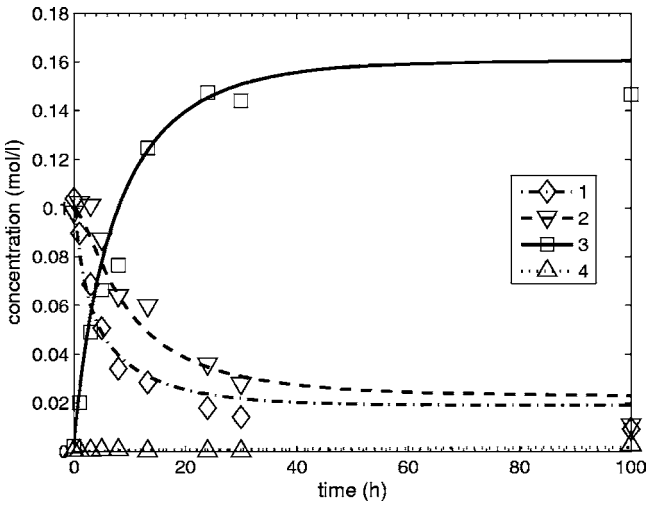

Figure 2. **Modelled reaction course and measured points for a reaction carried out with 0.2 mol/L substrate (0.2 mol/L, DL-phenylalanine ethyl ester; 2.5 mol %, 3,5-dinitrosalicylaldehyde; 1 vol %, Alcalase, pH 7.5; 25 °C; acetonitrile/water, 1:1).**

The catalyst deactivation has no influence on the optical purity of the product in this case because the enzyme loses its activity faster than the racemisation catalyst. Hence, the ratio k_{rac}/k_S is even increasing in the course of the reaction.

Optimisation. Having developed a model that describes the complete reaction course of the dynamic kinetic resolution, we considered an optimisation of the reaction. The determined values for E and k_{rac}/k_S show that an enhancement of the optical purity of the product is rarely possible because these parameters are already well adjusted (compare Figure 1). Because of the catalyst deactivation, a yield of 100% is as well difficult to reach, but variation of the reaction conditions (substrate and catalyst concentrations) showed that an increase of one of the catalyst concentrations by the factor of 5 increases the yield from 80% to 90%. In case of an increased Alcalase concentration the reaction time is as well shortened from 30 to 24 h. The shorter reaction time and higher yield increase the volumetric productivity by a factor of 1.4. For economic reasons further reactions were carried

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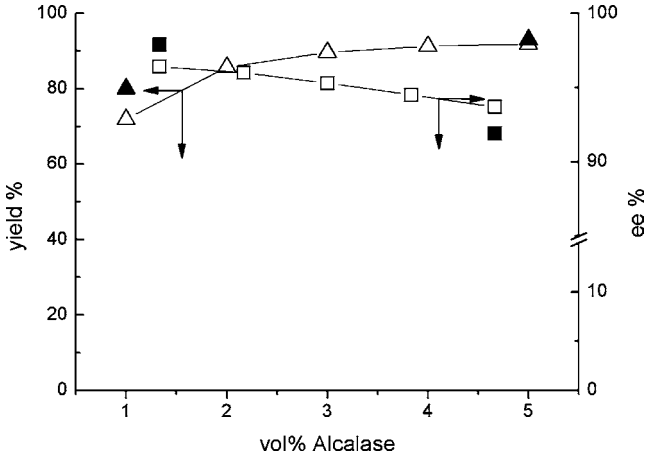

Figure 3. **Yield (triangles) and optical purity (squares) of the product as function of Alcalase concentration: modelled (open symbols) and experimental values (filled symbols).**

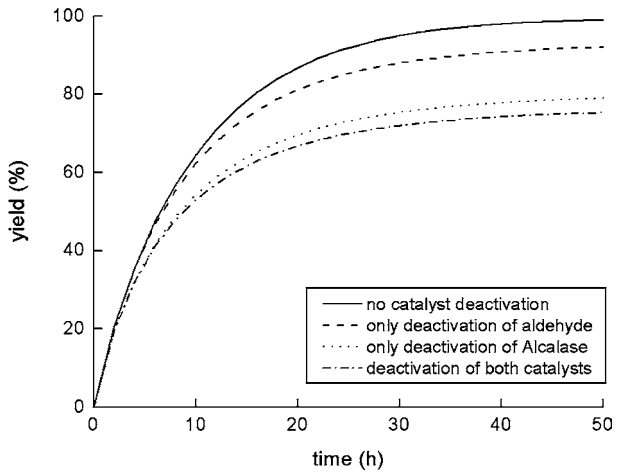

Figure 5. **Modelling of the reaction course with only one catalyst deactivated at a time shows that the obtainable conversion depends on the deactivation of catalysts (conditions as in Figure 2).**

out with 5-fold Alcalase-concentration (based on catalogue prices, under standard conditions dinitrosalicylaldehyde contributes to the catalyst costs with 97%, while Alcalase is only about 3%[27]). The depletion of optical purity due to a decrease of k_{rac}/k_S by change of the Alcalase concentration is about 6%, which corresponds very well with the theoretical value (compare Figures 1 and 3). However, there is another factor limiting the increase in Alcalase concentration. Besides the loss of optical purity of the product a further increase would not result in an appreciable increase of yield (Figure 3). Figure 3 also demonstrates a good agreement of experimental and calculated values.

Investigation of the reaction rates indicated that the increased conversion and shorter reaction time are due to a much higher effective formation rate of L-phenylalanine ethyl ester in the beginning of the reaction when the Alcalase concentration is increased (Figure 4). The effective formation rate is defined as

$$v_{eff} = v_r^+ - n_r^- \tag{6}$$

with the formation rate of L-phenylalanine ethyl ester by racemisation v_r^+ and the formation rate of D-phenylalanine ethyl ester by racemisation v_r^-. Because of the higher effective formation rate with increased Alcalase concentration the rate of enzymatic hydrolysis v_{enz} of **1** is much faster as well. Most of the reaction is completed after a few hours so that the effect of the catalyst deactivation is declining.

To establish further optimisation strategies, the role of catalyst deactivation was investigated. By simulation of the reaction with only one of the catalysts deactivated—which can easily be done having established the model—it can be investigated easily which one is more important—the deactivation of the chemocatalyst or of the enzyme. The data shown in Figure 5 prove that the deactivation of the enzyme is in fact the limiting factor in this reaction. Without any deactivation the theoretically reachable conversion of 100% is reached after 48 h. With deactivation of both catalysts only about 80% are predicted, which is reached by the experiments described earlier. Considering which catalyst deactivation affects the system most and therefore has to be improved in the first place, we simulated the reaction course with only one catalyst deactivated at a time. The data are displayed in Figure 3 as well. When the enzyme deactivation

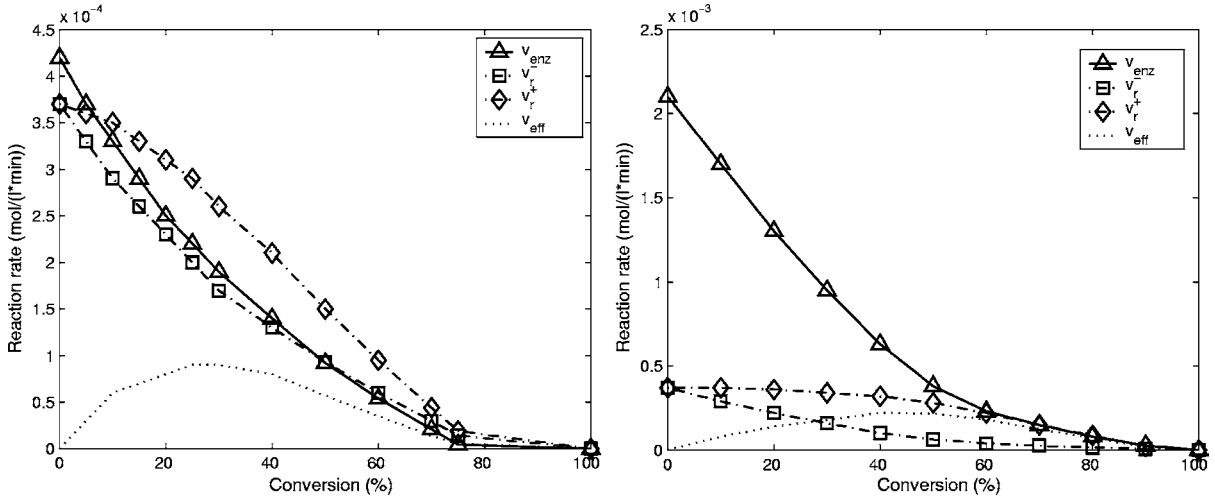

Figure 4. **Development of reactions rates of racemisation and cleavage and effective formations rate in the course of the reaction (left: conditions as in Figure 2; right: 5-fold Alcalase concentration); note different scaling of *y*-axis.**

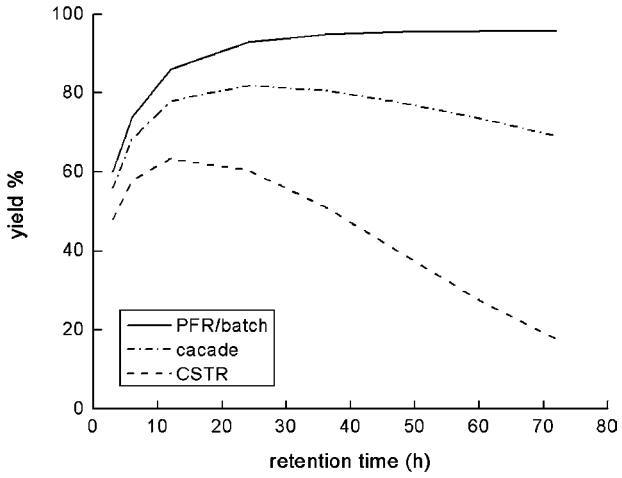

Figure 6. **Calculated conversion in different continuously operated reactors (feed/initial concentrations: 0.2 mol/L, DL-phenylalanine ethyl ester; 2.5 mol %, 3,5-dinitrosalicylaldehyde; 5 vol % Alcalase. Conditions: pH 7.5, 25 °C, 1:1 acetonitrile/water).**

is taken into account, the achievable conversion is insignificantly higher than with both catalysts deactivated, whereas a conversion of 95% is obtainable when only the salicylaldehyde is losing activity during reaction.

As expected, the optical purity of the product decreases slightly (1% ee) if only the racemisation catalyst is subject to catalyst deactivation because k_{rac}/k_S decreases. If the enzyme is subject to deactivation as well, the effect on the optical product purity is negligible (<0.5% ee). As a consequence, a continuous or batch-wise dosing of the Alcalase could be considered. This will reduce the amount of enzyme by a factor of 2, but as the enzyme is relatively cheap, as described before, this was not investigated further.

Continuously Operated Reactors. With the determined kinetic constants it was possible to simulate the reaction course of the DKR in continuously operated reactors as well. The concentrations calculated for steady state in an ideal working CSTR, a three-step cascade and plug flow reactor (PFR) allowed the determination of accomplishable yield and optical purity. The results are shown in Figure 6. The highest yield can be obtained in a PFR or batch reactor. In the CSTR and the cascade the course of the graph shows a maximum. First the yields rise with increasing retention time, but after about 18 h the catalyst deactivation becomes an important factor, and the yields decline. The enantiomeric excess calculated with the model is not shown because small deviations between calculated and real concentrations of the enantiomers lead to noteworthy variations in the ee value. In this calculation recovery of the catalysts was not taken into account.

(27) Alcalase (Merck, Darmstadt) 57 Euros/500 mL; 3,5-dinitrosalicylaldehyde (Aldrich, Steinheim), CAS number 2460-59-5, 150, 50 Euros/5 g.

4. Conclusions

A model describing the complete reaction course of a dynamic kinetic resolution was developed. Kinetic investigations and modelling showed that the deactivation of catalysts was the prominent constraint in reaching 100% conversion with high optical purity of the product, as can be achieved with DKR theoretically. Nevertheless, optimisation leads to an improvement in yield of 10% and a shortening of reaction time by a factor of 2.

Simulation of the reaction with only one catalyst deactivated at a time revealed that with respect to yield and productivity the enzyme should be the first target for further optimisation. Further work will therefore focus on a screening of commercially available proteases catalysing the same reaction. If this will not be successful, development of an enzyme with improved stability by directed evolution could be a target. However, it should be kept in mind that for a final judgment also the process economies—in this case the product specific catalyst consumption of both catalysts—have to be considered.

Modelling the reaction course in continuously operated reactors showed that batch and PFR respectively are the best reactor types to obtain high yield and optical purity in dynamic kinetic resolutions.

Due to the progress in fermentation methods and genetic engineering the method of choice for production of L-phenylalanine is certainly a fermentation process using microorganisms. However, for non-natural amino acids or amino acids having the other stereochemistry a process as described here might be the better choice. Our study demonstrates what are the limiting factors and how kinetic studies can be helpful for designing a better process.

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