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Lipase-Catalyzed Acylation in a Continuous Down-Flow Fixed-Bed Reactor[1]

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Abstract—The kinetic resolution of racemic 1-phenylethanol with ethyl acetate was investigated in a down-flow fixed-bed reactor operated in a continuous mode mainly at the molar ratio of 1 : 3 in 400 mL toluene at 70°C. The catalytic activity of the immobilized lipase was studied by: (i) changing the flow rates, (ii) utilizing different substrate concentrations, (iii) applying step changes using ethyl acetate, ethyl benzene, acetic acid, acetophenone etc., (iv) investigating the inhibitory effect of either the desired or the stoichiometric products (*R*)-1-phenylethyl acetate and ethanol, respectively), (v) elucidating the effect of water on the activity and stability of the immobilized lipase. The residence time distribution and the reactor hydrodynamics were also discussed along with kinetic modelling. The results were linked to the one-pot reactions.

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INTRODUCTION

The preparation of chiral drugs as single enantiomers via enantioselective acylation catalyzed by lipases is widely used in pharmaceutical synthesis [1–3]. Enantiopure compounds are efficiently produced by lipase catalyzed kinetic resolution of enantiomers giving high regio- and enantioselectivities in mild reaction conditions with multitude of substrates [4–8]. The two enantiomers of a racemic mixture react at different rates and one of the enantiomers is then transformed to the desired product while the other remains unchanged. After reaction, the mixture is enriched in the slower-reacting enantiomer.

The enantioselectivity is often given as enantiomeric excess of the products, P ($ee = \left(\frac{x_{P_R} - x_{P_S}}{x_{P_R} + x_{P_S}}\right) \times 100$) [9], which should be preferable close to 100%. Additionally, the enantiomeric ratio $E = \frac{k_{S_R}}{k_{S_S}}$ is given by the ratio of the rate constants for two enantiomers in the substrates, S. A value of E, which should preferable be above 50, can also be calculated from the following equation:

$$E = \frac{\ln\left[(1 - X(1 + ee(P)))\right]}{\ln\left[(1 - X(1 - ee(P)))\right]}, \quad (1)$$

in which X is the conversion [10].

Several organic compounds are water-unstable and/or water-insoluble and thus their synthesis is carried out in organic solvents [11]. Enzymes in non-aqueous solvents catalyze numerous reactions such as esterifications, transesterifications, aminolysis, while on the contrary these processes are completely suppressed by hydrolysis in water [12]. Different solvents can strip off the essential water from the catalyst in different ways [13, 14]. Essential water, which is the water around the enzyme to preserve the 3D structure of the protein in a catalytically active form, acts as a molecular lubricant to add protein a greater flexibility [13]. Typically enzyme activity decreases with increasing the polarity of the organic solvent [15]. Solvent hydrophobicity, characterized by the log$P$ value, indicates the logarithm of the partition coefficient in an octanol-water system [16]. The solvents with a log$P < 2$ are not favorable for enzymatic systems, because they strongly distort the water-enzyme interaction, which is essential for the enzyme activity, whereas solvents with a log$P$ between 2 and 4 are weak water distorters and can affect enzymatic activity in an unpredictable manner. Biocatalyst remains in active state in solvents with a log$P > 4$, which are not able to distort the water-enzyme interactions [17].

A few hydrolase-catalyzed enantioselective processes have been carried out in continuous flow systems [18–20]. Kinetic resolution of racemic alcohols and vinyl acetate with the molar ratio of 1 : 2 over an immobilized lipase was performed in a continuous-flow reactor at a 0.70 mL min^{-1} flow rate under 13 MPa of supercritical CO_2 ($scCO_2$) [21]. The *ee* of the optically active acetates was 99% and the productivity of the desired product was improved by over 400 times in a continuous-flow reactor compared to the corresponding batch reactions using $scCO_2$ [21, 22]. Continuous

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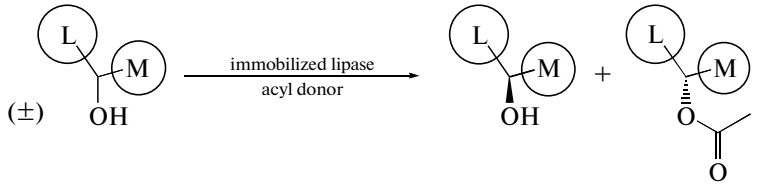

Fig. 1. Lipase catalyzed kinetic resolution following the Kazlauzkas' rule [6] of secondary alcohols.

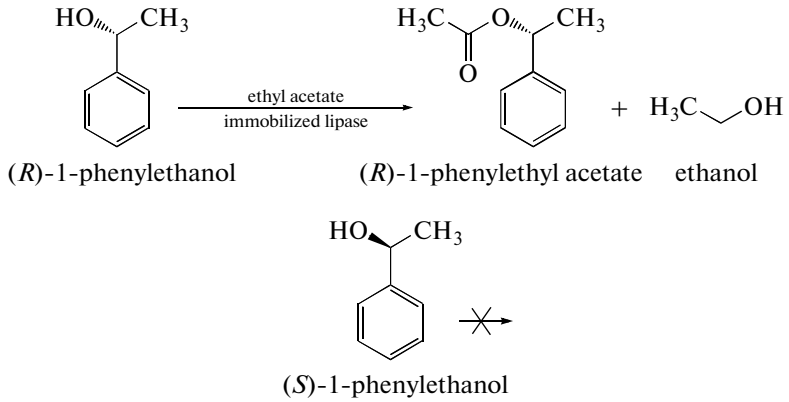

Fig. 2. Reaction scheme of enantioselective enzymatic kinetic resolution of racemic 1-phenylethanol with ethyl acetate over immobilized lipase.

packed-bed reactors offer several advantages including easy control and operation, reduction of labor costs, etc. [23, 24]. Promising results have been obtained in lipase-catalyzed kinetic resolution in continuous-flow packed-bed bioreactors [25] showing that the productivity was considerably enhanced in a continuous flow reactor compared to the corresponding batch mode.

The aim of this work was to study transient kinetics in the kinetic resolution of racemic 1-phenylethanol with ethyl acetate over lipase catalysts in a down-flow continuous reactor. The effect of acetophenone, ethyl benzene, acetic acid, water and ethanol on the catalytic behavior was studied, since these compounds are present in one-pot synthesis of *R*-1-phenylethyl acetate [26]. The main studied parameters were flow rates (and thus residence time), substrate concentrations. The continuous flow reactor and the batch reactor modes for the kinetic resolution of racemic 1-phenylethanol were compared.

EXPERIMENTAL

The SEM images of fresh and spent immobilized lipase catalysts were taken with the scanning electron microscope system Leo Gemini 1530 with a Thermo Scientific UltraDry Silicon Drift Detector (SDD) equipped with SE (secondary electron) and BSE (backscattered electron) detectors including an In-Lens detector.

The kinetic resolution of racemic 1-phenylethanol (Fig. 2) with ethyl acetate was performed in a down-flow continuous reactor (Fig. 3). The internal diameter and the length of the reactor are 1 cm and 12 cm, respectively. The reactor was filled with 3.5 cm quartz sand (>355 μm) on the top of a 1 cm quartz wool layer, located at the bottom. Above this quarz sand layer, the catalyst bed (2.25 cm), containing a mixture of 0.125 g immobilized lipase, Novozym 435, and 1.5 g quartz sand of a height of 2.9 cm, was placed. The uppermost quartz layer which dimension is 3.5 cm in length distributed the liquid flow above the catalyst bed. In a typical experiment, the kinetic resolution of racemic 1-phenylethanol (**rac**-1) with ethyl acetate (EA) was performed at the molar ratio of 1 : 3 in 400 mL toluene (J.T. Baker, 99%) at 70°C with 3 mL min^{-1} flow rate at atmospheric pressure. The liquid phase containing substrates was saturated with argon prior pumping into the reactor. The catalyst bed was washed with toluene at room temperature for 15 min before and after each experiment. The immobilized lipase was kept at +4°C overnight between the experiments.

The samples were analyzed with a gas chromatograph equipped with a FI detector and a chiral column (Chirasil Dex (250 μm × 0.250 μm × 25 m) and the following temperature programme was applied: 100°C (1 min)–0.30°C/min–130°C–15°C/min–200°C (10 min). Injector and detector temperature was 280°C and the split

ratio of 100 : 1 was applied. Acetophenone was used as an internal standard for quantitative analysis.

Residence time distribution was investigated by applying step changes using either ethanol (Etax Aa, 99.5%) or hexane (J.T. Baker, 99%) or ethanol and hexane in toluene. The experiments were performed at room temperature (25°C). In order to suppress possible evaporaion of the corresponding compounds the experiments with (i) 0.06 M ethanol, (ii) 0.06 M hexane or, (iii) 0.06 M ethanol and 0.06 M hexane mixture were performed. Toluene was pumped to the reactor for 10 min before and after the experiments. The samples collected both from the inlet and outlet streams of the reactor, were taken every 30 s to obtain informative data. The effect of different flow rates was investigated by applying flow rates ranging from 1.5 to 3 mL min^{-1}.

Different concentrations of substrates were also studied. The ratio of **rac**-1 (Acros, 97%) to ethyl acetate (Sigma-Aldrich, >99.5%) was changed from 1 : 6 to 1 : 1 whereas the flow rates were kept constant. The effect of different substrate concentrations was also investigated at different volumetric flow rates. The concentrations of **rac**-1 in the first and second experiments were 0.02 and 0.04 M whereas 0.06 and 0.12 M EA were used, respectively.

The effect of acetophenone (Acros, 99%), ethyl benzene (Aldrich), acetic acid (J.T. Baker, 99–100%) and water on the activity and stability of the immobilized lipase was investigated. Moreover, the inhibitory effects of either the desired or the stoichiometric products ((*R*)-1-phenylethyl acetate (Acros, 96%) and ethanol (Etax Aa, 99.5%), respectively) were studied as well. The concentrations of **rac**-1 and ethyl acetate were kept constant, being 0.02 and 0.06 M, respectively. After the start up of the reaction, the known concentration of the compounds to be investigated (0.02 M) was added to the known concentration of the liquid phase at certain time intervals.

The activity of the immobilized lipase was determined by comparing the activities obtained over fresh and spent catalysts. The experiments were performed using 0.02 M **rac**-1 and 0.06 M ethyl acetate in 400 mL toluene with 3 mL min^{-1} flow rate. The very same experiments were carried out 30 days after the first experiment. SEM images of the fresh and spent catalysts were taken in order to make visible morphological changes which could be used to rationalize activity decrease.

RESULTS AND DISCUSSION

The hydrodynamics of the reactor system was studied by following the responses to step changes in the feed concentrations. The deviation from plug flow was then evaluated by performing a rough time domain fit to measured concentration responses, such as that shown in Fig. 4. Since there was some noise in the experimental data and only a limited amount of data points was available a simple one-parameter mixing cell approximation (tanks-in-series model, TIS) was used to model the reactor setup. The residence time distribution for the tanks-in-series is given by

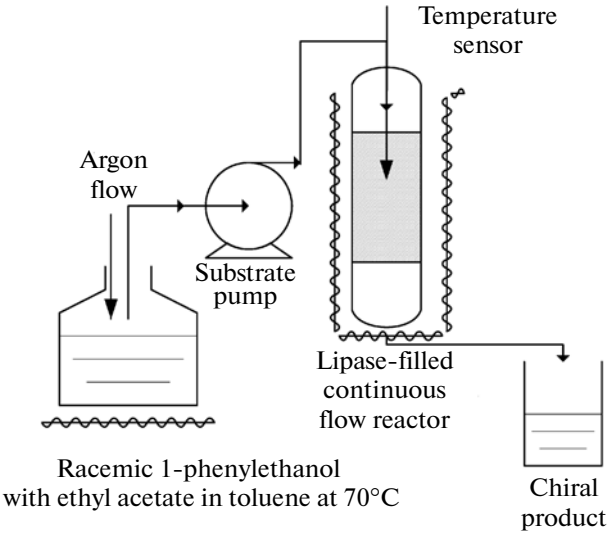

Fig. 3. Simplified illustration of the experimental apparatus (~~: temperature control unit).

$$F(t) = \frac{c}{c_0} = 1 - e^{-nt/\bar{t}}\left[\sum_{i=1}^{n}\frac{(nt/\bar{t})^{i-1}}{(i-1)!}\right], \quad (2)$$

and the corresponding washout function, $W(t)$, is obtained from the usual relationship

$$W(t) = 1 - F(t). \quad (3)$$

In order to get a satisfactory description (Fig. 4) of the measured hydrodynamics six mixing cells were used with a mean residence time of roughly 3 min, which is more than three times longer than the residence time calculated based on reactor volume and volumetric flow. This is a strong indicator that the pump and inlet lines account for the main total volume of the studied system. The suggestion was verified in a separate experiment, in which it was found that the mean residence time from feed vessel to reactor inlet was approximately 2 min for the same experimental conditions. The degree of back-mixing in the actual reactor bed was roughly the same as in the upstream apparatus.

The number of mixing cells in the TIS model can directly be related to the Peclet number, *Pe*, for the one dimensional axial dispersion model:

$$n = \frac{Pe^2}{2\left(Pe - 1 + e^{-Pe}\right)}. \quad (4)$$

Five to six mixing cells in the tanks-in-series model correspond to Peclet numbers of about 10 in the axial dispersion model. In other words, in the current reac-

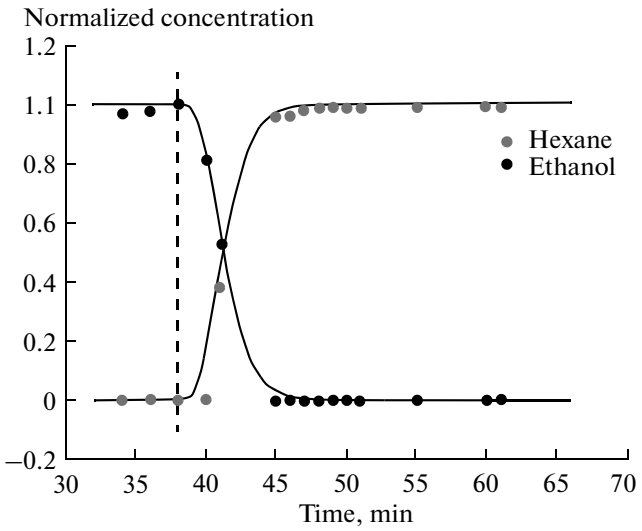

Fig. 4. Transient kinetic by applying step changes at 25°C using $\dot{V}$ =3 mL min^{-1}. Notation: *1*, 0.06 M of ethanol in toluene; *2*, 0.06 M of hexane in toluene.

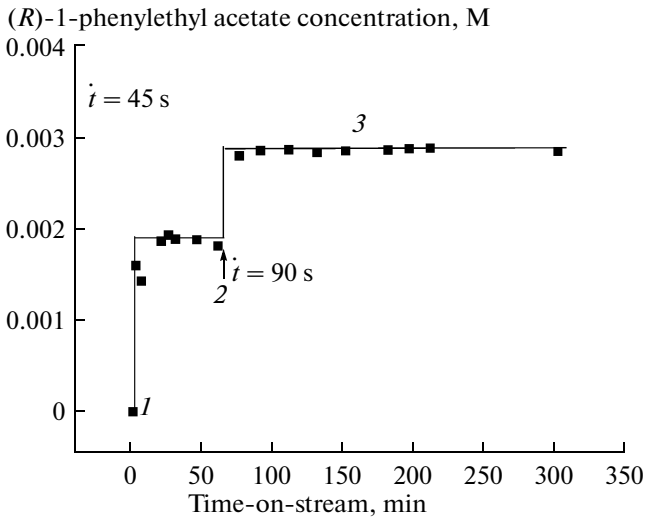

Fig. 5. Kinetic resolution of **rac**-1 at 70°C. Notation: *1*, start-up of the first experiment using $\dot{V}$ = 3 mL min^{-1}; *2*, start-up of the second experiment using $\dot{V}$ =1.5 mL min^{-1} was applied; *3*, using $\dot{V}$ = 1.5 mL min^{-1}. The ratio between the concentrations of the **rac**-1/EA is 1 : 3.

tor a flow pattern is present in which convective transport of mass (plug flow) in the system dominates over dispersive transport by roughly one order of magnitude. The deviations from ideal flow for the investigated system are small enough that the results shown in Figs. 5–12 can be attributed to intrinsic kinetic and possibly adsorption effects.

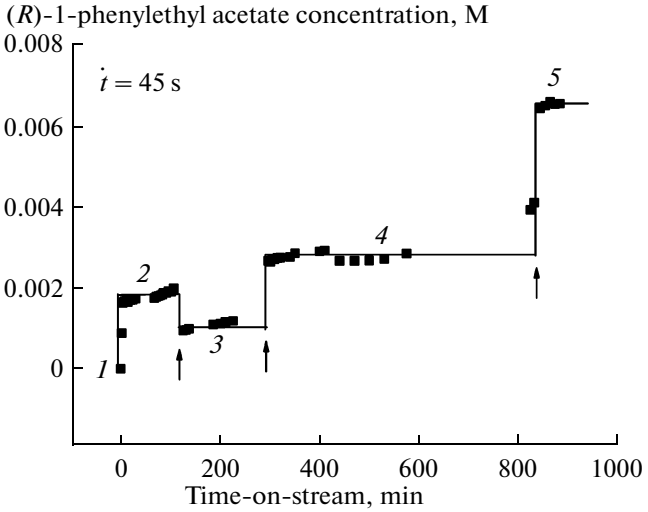

Fig. 6. Kinetic resolution of **rac**-1 at 70°C. The effect of different concentrations of substrates. Notation: *1*, start-up of the first experiment using $\dot{V}$ = 3 mL min^{-1}; *2*, the ratio between racemic 1-phenylethanol (**rac**-1) to ethyl acetate (EA) (**rac**-1/EA) is 1 : 3; *3*, the ratio between **rac**-1/EA is 1 : 1; *4*, the ratio between **rac**-1/EA is 2 : 3; *5*, the ratio between **rac**-1/EA is 1 : 6. The arrows (→) show when the different concentrations were applied at time-on-stream.

The effect of the residence time ($\hat{\tau} = V/\dot{V}$, where V is the catalyst bed volume and $\dot{V}$ is the volumetric flow rate of the liquid, respectively) was investigated by applying different volumetric flow rates. Initially, the residence time was increased at 60 min time-on stream from 45 to 90 s. The results showed that doubling the residence time without changing the concentrations of substrates doubled (*R*)-1-phenylethanol conversion, as expected. The highest (*R*)-1-phenylethanol conversion was 30% after 300 min time-on-stream corresponding to 100% selectivity of the desired acetate ((*R*)-1-phenylethyl acetate). The (*R*)-1-phenylethyl acetate concentration was enhanced by 53% when the residence time increased two fold (Fig. 5). No deactivation of the immobilized lipase was observed.

Different concentrations of the substrates were also applied whereas the residence time was kept constant (Fig. 6). The start-up of the reaction was carried out with the ratio of **rac**-1 and ethyl acetate equal to 1 : 3 (0.02 and 0.06 M, respectively) for 180 min time-on-stream. The ratio of the substrates was changed from 1 : 3 to 1 : 1 (0.02 and 0.02 M, respectively) in the second step. Decreasing the concentration of ethyl acetate decreased the desired product concentration in half (Fig. 6) meaning that the excess of ethyl acetate which was used as an acyl donor in this reaction is necessary to obtain high yields of the desired product In the third step, **rac**-1 concentration was doubled, 0.04 M, comparing to the start-up concentrations whereas ethyl acetate concentration was kept constant (0.06 M). Doubling **rac**-1 concentration enhanced the concentration of (*R*)-1-phenylethyl acetate by 50% (Fig. 6). Compared to the second step, in the third step the concentrations of **rac**-1 and ethyl acetate increased

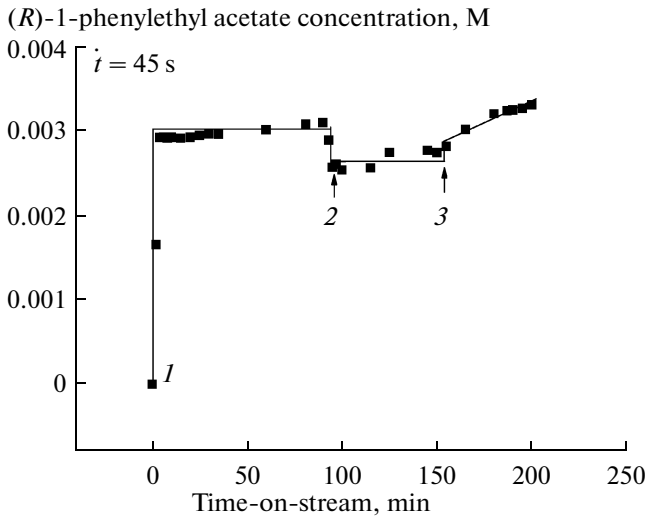

Fig. 7. Kinetic resolution of **rac**-1 at 70°C. Notation: *1*, start-up of the experiment using $\dot{V} = 3$ mL min^{-1} with **rac**-1/EA is 1 : 3; *2–3*, the effect of 0.02 M acetophenone addition to the reaction media between 90 to 180 min time-on-stream; *3*, acetophenone feed switch-off.

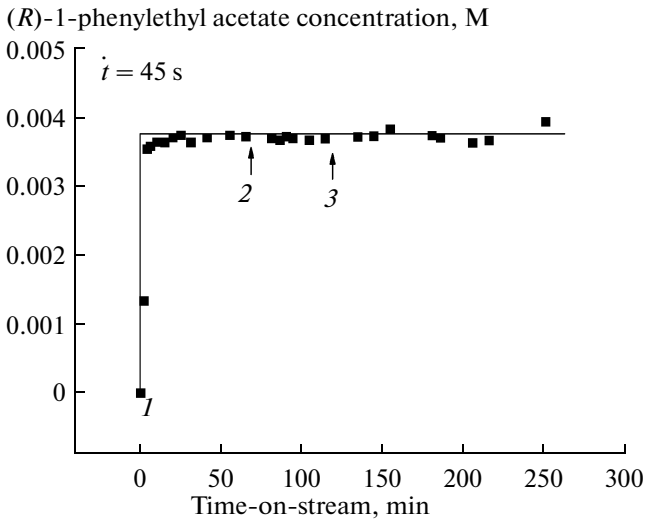

Fig. 8. Kinetic resolution of **rac**-1 at 70°C. Notation: *1*, start-up of the experiment using $\dot{V} = 3$ mL min^{-1} with **rac**-1/EA is 1 : 3; *2–3*, the effect of 0.02 M ethyl benzene addition to the reaction media between 60 to 120 min time-on-stream.

from 0.02 and 0.02 M to 0.04 and 0.06 M, respectively meaning that the ratio of **rac**-1 to ethyl acetate was decreased from 1 to 0.666. Increased concentrations of substrates increased (*R*)-1-phenylethyl acetate concentration by 200% (Fig. 6). Finally, the ratio of **rac**-1 to ethyl acetate was changed to 1 : 6 (0.02 and 0.12 M, respectively). Very high excess of ethyl acetate gave the highest desired product concentration (Fig. 6).

Generally, it can be stated that in experiments with step changes the enzyme was the same in all experiments. However, enzyme activity can change with time and furthermore, there are several effects, which can influence the enzyme acitivity, such as water content. Accordingly, the results presented in Figs. 7–12 should be compared only within the course of a single pulse experiment and not with each other. To illustrate this point, we can compare the concentration of the (*R*)-1-phenylethyl acetate with the fresh catalyst in the step 1 in Fig. 5 with the results obtained under similar conditions in Figs. 7–12. It can be seen that the former concentration is 0.002 M (Fig. 5, step *1*), whereas it was about 0.003 M or even more in the initial step in experiments including addition of acetophenone (Fig. 7), ethyl benzene (Fig. 8), acetic acid (Fig. 9), or on changing the ratio of **rac**-1 to ethylacetate (Fig. 10), and adding the product (Fig. 11) and water (Fig. 12).

When 0.02 M of acetophenone was added to the reaction media between 90 to 180 min time-on-stream at 70°C passed with a flow rate of 3 mL min^{-1}, the results showed that acetophenone slightly inhibited the lipase activity (Fig. 7). The desired product concentration decreased by 17% after adding 0.02 M acetophenone.

The effect of ethyl benzene on the enzyme activity and stability was also studied. Since ethyl benzene was observed as the major side product in the one-pot reactions due to the hydrogenolysis of racemic 1-phenylethanol, 0.02 M ethyl benzene addition to the reaction media at 60 until 120 min time-on-stream was studied in the continuous flow system. The results indicated that presence of ethyl benzene did not retard the acylation activity of lipase (Fig. 8).

The highest inbitory effect was observed with 0.02 M acetic acid addition between 120 to 180 min time-on-stream (Fig. 9). Acetic acid was produced by hydrolysis of ethyl acetate with water generated in a side reaction of racemic 1-phenylethanol and dehydration over palladium supported on acidic supports in one-pot reactions [26]. Enzymes are very sensitive to pH changes since pH significantly influences the secondary, tertiary and quaternary structure of proteins thus affecting enzyme deactivation kinetics [27]. The enzyme inactivation rate, as expected, in most cases is significantly dependent upon the pH of the solution [28]. However, the definition of pH measured in water cannot be applied to aqueous mixture of organic solvents since pH has no meaning in organic solvents [28]. It is well known that the enzymatic activity in organic solvents is related to the protonation state of enzyme's ionizable groups reflecting the pH of the last aqueous solution where they were lyophilized [29]. This phenomenon is called the pH memory. The

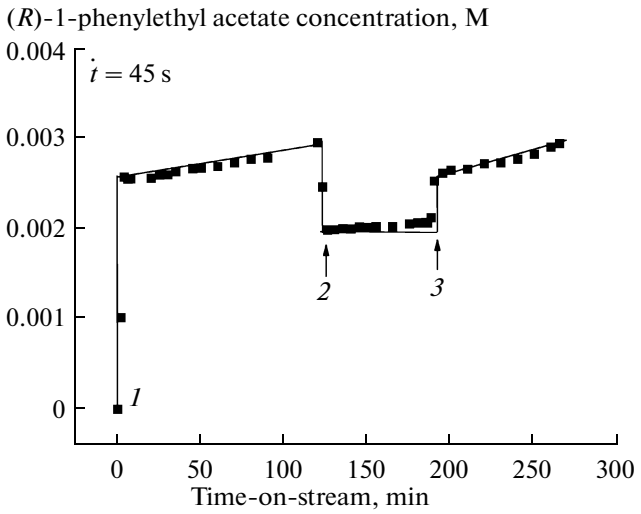

Fig. 9. Kinetic resolution of **rac**-1 at 70°C. Notation: *1*, start-up of the experiment using $\dot{V} = 3$ mL min^{-1} with **rac**-1/EA is 1 : 3; *2–3*, the effect of 0.02 M acetic acid addition to the reaction media between 120 to 180 min time-on-stream.

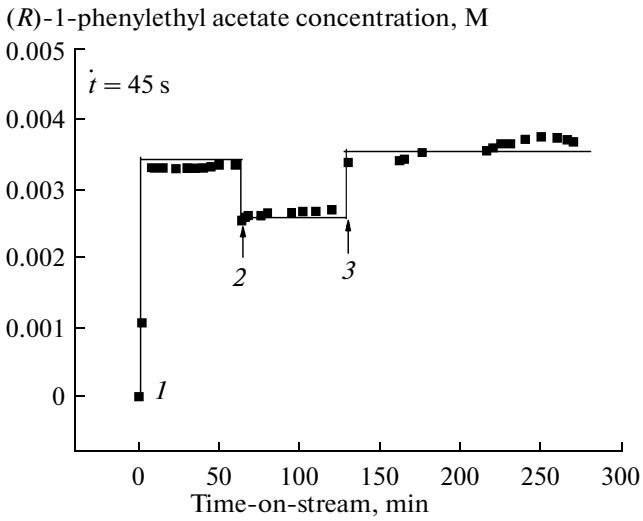

Fig. 10. Kinetic resolution of rac-1 at 70°C. Notation: *1*, start-up of the experiment using $\dot{V} = 3$ mL min^{-1} with **rac**-1/EA is 1 : 3; *2–3*, the effect of 0.02 M (*R*)-1-phenylethyl acetate addition to the reaction media between 60 to 120 min time-on-stream.

results obtained from acetic acid addition (Fig. 9) are in line with the influence of pH changes over enzymes.

One of the most often considered issues in the literature related to the activity and stability of enzymes is the product inhibition. The influence of the desired product (*R*)-1-phenylethyl acetate and stoichiometric product ethanol on the lipase activity was studied. A portion of 0.02 M of (*R*)-1-phenylethyl acetate was fed to the reaction media at 60 min time-on-stream and the feed continued for 60 min. The desired product concentration formed in the reaction decreased by 30% indicating that enzyme activity decreased due to the high product concentration (Fig. 10). This result show that the product should be withdrawn from the reaction media continuously to achieve high desired product yields.

Ethanol is stoichiometrically produced during the kinetic resolution of (*R*)-1-phenylethanol with ethyl acetate over lipase. When 0.02 M ethanol was added to the reaction mixture (Fig. 11, step *2*), the concentration of the desired product decreased by almost 100% (Fig. 11). The inhibition of lipase due to high concentration of ethanol can be explained by the influence of hydrophilic solvents on the enzyme activity in organic solvents. The enzyme activity usually decreases with increasing the hydrophilicity of the solvent [15] because hydrophilic solvents apt to strip off essential water which is needed by enzyme to preserve the 3D structure of the protein in a catalytically active form [8]. The active site of the enzyme intends to disperse resulting in unfolding of the molecule [7] without the essential water around. The decrease in the activity of lipase is consistent with the literature since ethanol is one of the most hydrophilic organic solvents with—$\log P = 0.24$ value [30]. Another reaction, which could in principle occur when excess of ethanol is added, is ethanolysis of (*R*)-1-phenylethyl acetate. The extent of ethanolysis is difficult to quantify, since the *ee* of (*R*)-1-phenylethyl acetate remained constant during the reaction being about 98% and traces of (*S*)-1-phenylethyl acetate were observed in the product mixture. Furthermore, the corresponding *E* value was ca. 100. An increase in the desired product concentration after the withdrawal of ethanol from the reaction media at point *3* (Fig. 11) was observed. The activity of the immobilized lipase enhanced because the liquid phase was replaced by the fresh substrate mixture.

Essential water is needed to keep the enzyme active even though the reaction media contains mainly organic solvent and/or substrates. On the other hand, water in the reaction mixture can cause unfavourable equilibrium in reversed hydrolysis and lead to hydrolytic side reactions in transferase catalyzed reactions. It has been shown that the water content in the reaction mixture is best described in terms of water activity [31]. A quick and more convenient way to determine the water activity is to measure water concentration [32]. There are several reports in the literature on the effect of water activity on the catalytic behavior of lipases in organic media [33], and it is clear that different enzymes have quite different requirements for water activity. Lipases can be active at water activities very close to 0 [34].

The water effect was in the focus of interest of the present study. In order to investigate the effect of excess water, water was added to the reaction media

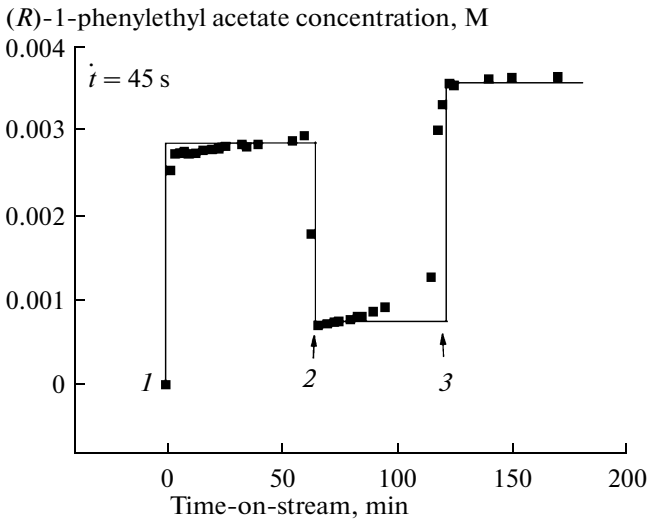

Fig. 11. Kinetic resolution of **rac**-1 at 70°C. Notation: *1*, start-up of the experiment using $\dot{V} = 3$ mL min^{-1} with **rac**-1/EA is 1 : 3; *2–3*, the effect of 0.02 M ethanol addition to the reaction media between 60 to 120 min time-on-stream.

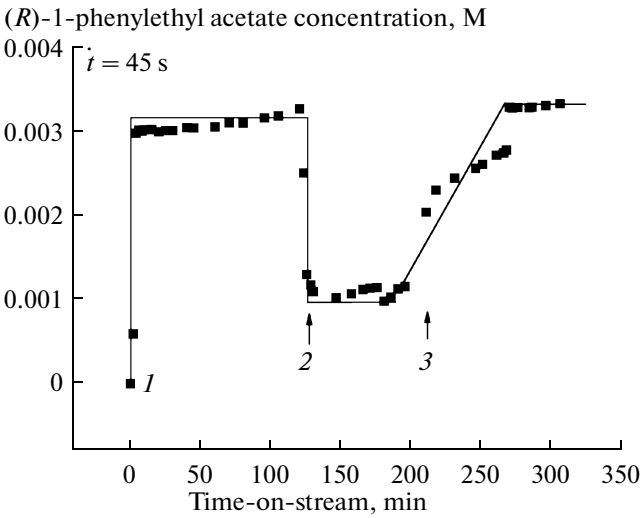

Fig. 12. Kinetic resolution of **rac**-1 at 70°C. Notation: *1*, start-up of the experiment using $\dot{V} = 3$ mL min^{-1} with **rac**-1/EA is 1 : 3; *2–3*, the effect of 0.02 M water addition to the reaction media between 60 to 120 min time-on-stream.

since it was formed as a result of a side reaction, dehydration of racemic 1-phenylethanol, due to the acidic support properties of palladium catalysts in batch reactor [26]. The results showed that the excess water (0.02 M) diminished enzyme activity significantly (Fig. 12). The desired product concentration decreased by 68% after the water addition. The decrease can be explained by the limited diffusion of the reactants to the active site of the enzyme. The reason for the gradual increase of the concentration of (*R*)-1-phenylethyl acetate before 275 min is unclear. After that time, the concentration of the product increased suddenly to about the same level, as it was before water addition. It should, however, be stated that there can be several reasons for this kind of behavior, for example the conformation of the enzyme can be changed as a function of water concentration. Since the excess water generates an extra layer around the enzyme and thus clogs the active site of the enzyme to interact with organic phase including reactants, the desired product formation decreased substantially. The inhibitory effect of water disappeared after water was removed from the inlet mixture (Fig. 12). The hydrolysis of the product back to (*R*)-1-phenylethanol and acetic acid might also occur. Analogously, as it was described above in the section describing ethanolysis, also in the case of product hydrolysis, the *ee* of the product remained close to 98% and traces of (*S*)-1-phenylethyl acetate were formed. Thus, it could be concluded that the addition of water can decrease the concentration of (*R*)-1-phenylethylacetate via making the reaction mixture more polar as well as hydrolyzing in some extent the product. The extent of hydrolysis was not determined in this work.

The activity of the immobilized lipase recorded as a function of time decreased by about 40% after 30 days (Fig. 13). The experiments performed with a fresh catalyst gave around 0.004 M desired product concentration, however, when the very same experiments were carried out over the 30 days spent catalyst, the desired product concentration dropped from 0.004 to 0.0025 M. It is interesting to note that the activity in a fixed bed reactor equal to 1.6×10^{-6} mol/s is very close to activity of an immobilized enzyme observed in a batch reactor [35]. The decrease in the catalytic activ-

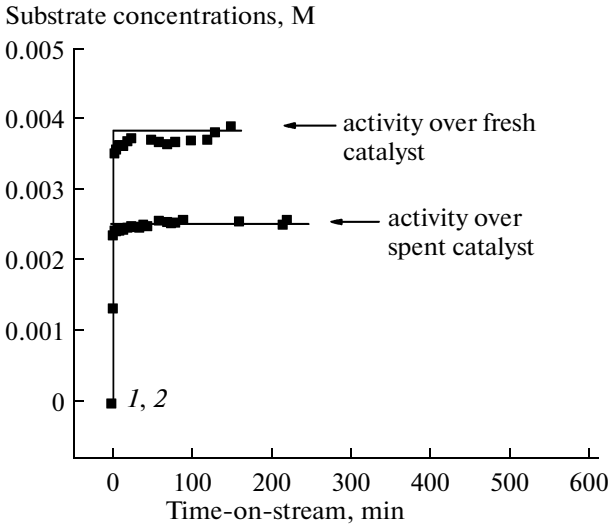

Fig. 13. Kinetic resolution of **rac**-1 at 70°C. Notation: *1–2*, start-up of the first and second experiment using $\dot{V} =$ 3 mL min^{-1} with **rac**-1/EA is 1 : 3.

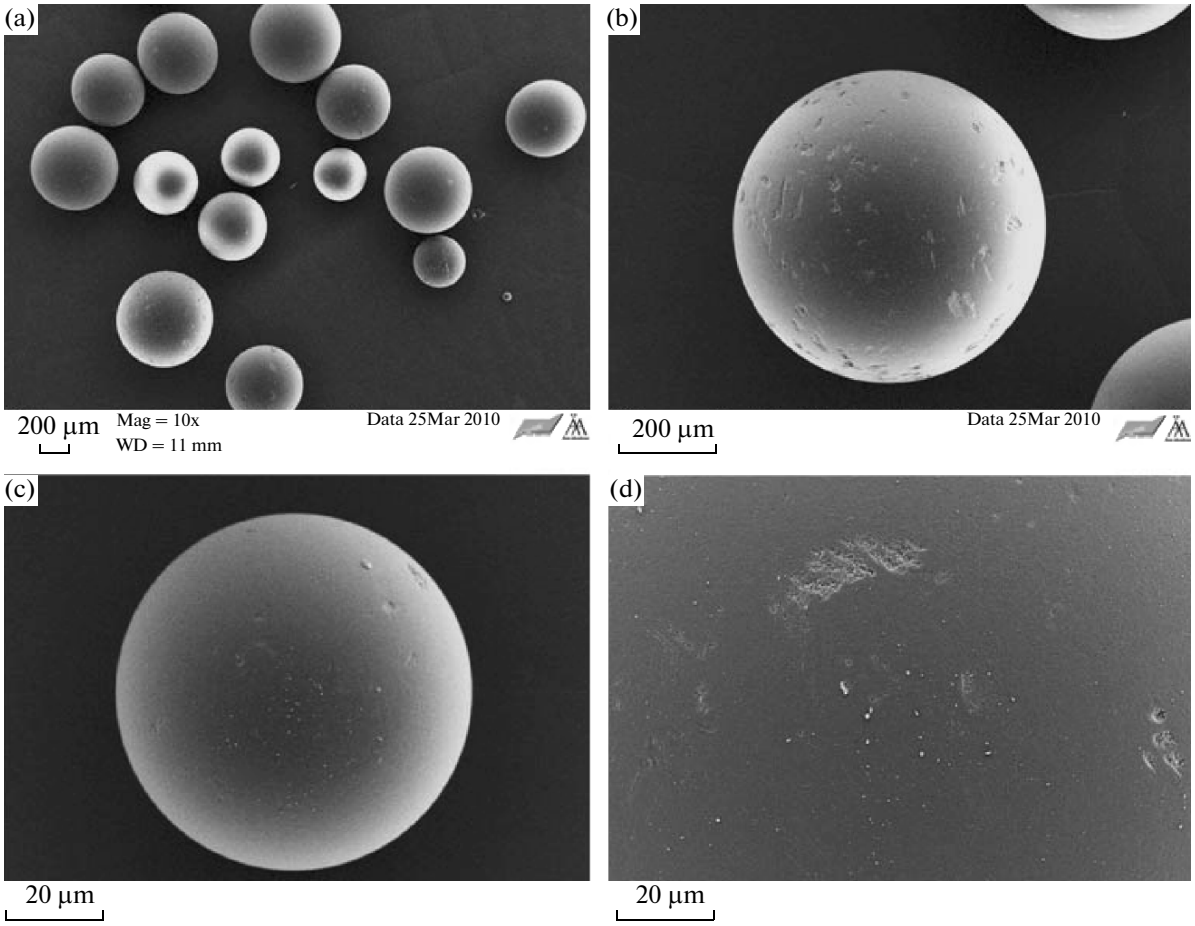

Fig. 14. images of the fresh immobilised lipase. (a) 30×, (b) 100×, (c) 250×, (d) 500×.

ity of the immobilized enzyme was also observed in the experiments conducted at different time intervals. The SEM pictures from fresh and spent catalysts clearly showed that the activity decrease was due to the morphological changes of the spent catalyst. The SEM pictures taken from the fresh catalyst (Fig. 14a) demonstrate smooth surface of the catalyst. The same pictures for the spent catalysts (Fig. 15a) give a clear image of the destruction of the catalyst integrity. The pictures taken with 200× and 500× magnififcation (Figs. 14c, 14d) show that the surface of the fresh catalyst is very smooth compared to the surface of the spent catalyst (Figs. 15c, 15d) indicating that the decrease of the enzyme activity might be a result of the physical changes of the immobilised enzyme during the reactions caused by organic solvents and/or substrates.

As indicated above the same reaction was previously studied by the authors in a batch reactor [35]. Kinetics in the batch reactor was discussed in detail in [35], thus the aim of the current work was to compare batch and continuous operation and to reveal influence of substrates present during one-pot synthesis on acylation. In kinetic modelling performed for the batch mode it was sufficient to describe kinetics without inclusion adsorption constants of reactants. Experiments in the continuous mode clearly highlighted influence of ethanol and the products, in particular they indicated that there is inhibition by the reacting phenvlalcohol, as well as the product and ethanol.

In [35] kinetic modelling revealed first order in ethylacetate thus in the present work also the first order was utilized resulting in the following kinetic equation

$$r = \frac{k_1 C_{\mathrm{EA}} C_{R-\mathrm{PE}}}{1 + K_{R\text{-PEAC}} C_{R\text{-PEAC}} + K_{R\text{-PE}} C_{R\text{-PE}} + K_{S\text{-PE}} C_{S\text{-PE}} + K_{\mathrm{EtOH}} C_{\mathrm{EtOH}}}, \quad (5)$$

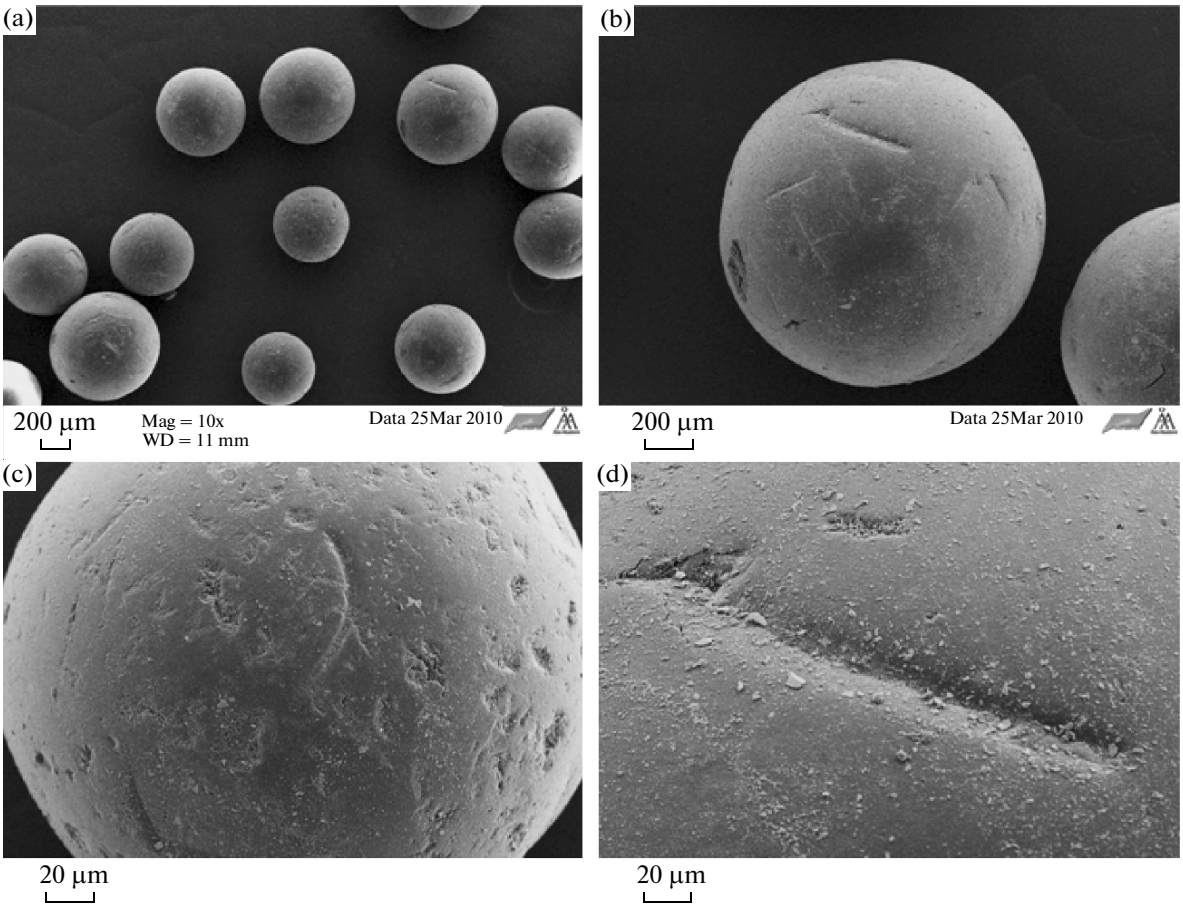

Fig. 15. SEM images of the spent immobilised lipase. (a) 30×, (b) 100×, (c) 250×, (d) 500×.

where *R*-PEAC, *R*-PE, *S*-PE and EtOH denote respectively (*R*)-phenylethylacetate ethylacetate, (*R*)- and (*S*)-1-phenylethanol as well as ethanol.

For the calculation of parameters, differential equation (5) describing the changes in the concentrations profiles of the reagents and products was solved by means of ModEst software [36]. Using Levenberg-Marquardt simplex method, the target function, which was defined as incompliance between the experimental and calculated values of concentrations was used to find the parameters. The sum of the residual squares between the model and the experimental data (Fig. 16) was minimized using the following objective function;

$$Q = \left\| x_{\text{exp}} - x_{\text{est}} \right\|^2 = \sum_t \sum_i \left(x_{\text{exp},it} - x_{\text{est},it} \right)^2, \qquad (6)$$

where x_{exp} is the experimental value and x_{est} denotes the predictions given by the model, i is the component index and t is the time value. The quality of the fit and accuracy of the model description was defined by the degree of explanation R^2;

$$R^2 = 100\left(1 - \frac{\left\| x_{\text{exp},i} - x_{\text{est},i} \right\|^2}{\left\| x_{\text{exp},i} - \overline{x}_{\text{est}} \right\|^2} \right), \qquad (7)$$

where $\overline{x}_{\text{est}}$ is the mean value of all the data points.

The results of the simulations for experimental data in Fig. 16 are given in table.

R^2, %	k_1, L mol^{-1} min	$K_{R\text{-PE}}$, L mol^{-1}	$K_{S\text{-PE}}$, L mol^{-1}	$K_{R\text{-PEAC}}$, L mol^{-1}	K_{EtOH}, L mol^{-1}
86	12.30 ± 0.58	490 ± 340	negligible	154 ± 59	1180 ± 405

The degree of explanation as high as 86% evidences a good compliance between the experimental data and the calculated values of the concentrations of the reagents. The fit of the kinetic model to the experimental data for the concentration profiles could be probably still improved taking into account activity increase with time. Despite the relatively large number of parameters in the proposed mechanism, the model

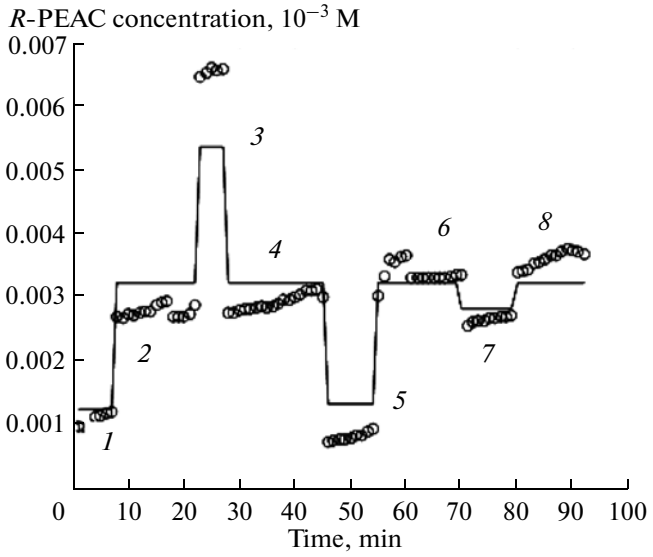

Fig. 16. Kinetic resolution of **rac**-1 at 70°C. The effect of different concentrations of reactants. Concentration of (*R*)-1-phenylethanol and (*S*)-1-phenylethanol was always 0.01 M each. Concentrations of other reactants were *1*: EA = 0.02 M, *2*: EA = 0.06 M, *3*: EA = 0.12 M; *4*: EA = 0.06 M; *5*: EtOH = 0.02; *6*: EA = 0.06 M; *7*: EA = 0.06 M; *R*-PEAC = 0.02 M; *8*: EA = 0.06 M.

was fitting the data with a good degree of accuracy. The model was able to rationalize lipase catalyzed acylation in a continuous down flow fixed bed reactor.

CONCLUSIONS

The utilization of the continuous flow reactor for the kinetic resolution of racemic 1-phenylethanol with ethyl acetate in toluene over an immobilized lipase was successfully demonstrated in this study. The results were discussed in connection to the acylation of racemic 1-phenylethanol in toluene over immobilized lipase utilizing one-pot system operated in batch mode. The effects of different residence times and substrate concentrations were investigated by applying different flow rates and ratios of **rac**-1to ethyl acetate, respeLctively. The results showed the absence of the external mass transfer.

The inhibitory effect of organic compounds used in the reaction such as ethyl acetate, ace lie acid, acelophenone etc., on the activity and stability of the immobilized lipase was also studied. No influence of excess ethyl benzene on the enzyme activity was observed whereas the enzyme activity was slightly inhibited by acetophenone. Ethyl benzene is the major side product formed in one-pot reactions due to the acidic character of the metal support. The formation of ethyl benzene occurrs *via* hydrogenolysis reactions of racemic 1-phenylethanol under H_2 flow. The water is released during the hydrogenolysis reactions and acetic acid is formed by ethylacetate hydrolysis. Therefore the effect of acetic acid on the activity of enzyme was also investigated since the inhibitory effect of acids on enzymes is very well known. The acid changes catalytically active 3D structure of enzymes causing the activity loss. The inhibition on the lipase activity due to acetic acid was clearly observed in this study. The experiments to elucidate the inhibitary effect of either the desired or the stoichiometric products ((*R*)-1-phenylethyl acetate and ethanol, respectively) with the inhibitory effect of water on the activity and stability of the immobilized lipase was also carried out. The slight inhibition due to the desired product was observed indicating that the product should be withdrawn continuously from the reaction media to increase the yield in the batch mode. The activity of lipase was significantly diminished by excess ethanol. The decreases of enzyme activity can be explained with the increase in the hydrophilicity of the solvent. Hydrophilic solvents apt to strip off essential water which is needed by enzyme to preserve the 3D structure of the protein in a catalyticaly active form. The similar effect was observed due to the increase of water concentration in the reaction media. The decrease in the activity of enzyme was also followed as a function of time. The desired product concentrations diminished over the catalysts used for 30 days. SEM pictures demonstrated that the spent catalysts are much more damaged whereas the fresh catalysts had smoother surface than the spent samples.

In kinetic modelling performed previously for the batch mode it was sufficient to describe reaction kinetics without including adsorption constants of the reactants. Experiments in the continuous mode clearly revealed influence of ethyl acetate, ethanol and the reaction product. Kinetic analysis of the experimental data obtained in the continuous mode was thus performed, taking into account a more complicated picture of adsorption, showing a good correspondence between experimental and calculated values.

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