Neat Lipase-Catalyzed Kinetic Resolution of Racemic 1-Phenylethanol and a Straightforward Modelling of the Reaction

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Abstract

Enzymatic kinetic resolution of racemic 1-phenylethanol catalyzed by immobilized *Candida antarctica* lipase B was investigated in a neat systems at the temperature range of 30-70°C. Synthetic triglycerides, namely glycerol triacetate and glycerol tributyrate, were applied as the esterification agents.

Both esterification agents were efficient regarding to the enantioselectivity (>1000). Initial rate of reaction and the kinetic constants were influenced by the applied esterification agent significantly. A detailed modelling approach is presented and verified in the temperature range on 30-60°C for the tributyrin system.

Keywords

Kinetic resolution, neat system, ping pong bi bi kinetics, activation energy of an enzymatic reaction, free enthalpy difference of an enzymatic reaction

1. INTRODUCTION

The preparation of enantiomerically enriched or preferably enantiopure products is one of the hot topics of research due to the different bioactivity and bioavailability of enantiomers. Several chemical methods are available for producing optically active chiral compounds (Siedlecka 2013; Fogassy et al. 2006). However, biological approaches as biocatalysis and

bioconversion are advantageous due to their high efficiency, mild reaction conditions, stereospecificity and low environmental impact (Johannes et al. 2006).

Enantiopure secondary alcohols are used as chiral building blocks and synthetic intermediates in asymmetric synthesis, pharmaceutical-, agrochemical- and fine chemical industries. Kinetic resolution of racemic secondary alcohols is an efficient method to obtain optically active alcohols (Pellissier 2011) preferably catalysed by lipases (Ghanem and Aboul-Enein 2004; Ghanem 2007; Singh et al. 2010). Lipases possess wide substrate specificity, moreover they do not require cofactors (Sharma et al. 2011; Jaeger and Eggert 2002; Kirk and Christensen 2002). Furthermore, lipases from *Candida antarctica* show high thermostability, they catalyse reactions in organic solvents, and also in non-conventional solvents like supercritical fluids and ionic liquids (Fan et al. 2011; Habulin and Knez 2009; Paljevac et al. 2009). A widely used commercial immobilized lipase is Novozyme 435.

Lipase-catalysed esterification of 1-phenylethanol is one of the best known model reaction of the kinetic resolution of secondary alcohols (Zhou et al. 2011; Overmeyer et al. 1999; Chua and Sarmidi 2004; Chua et al. 2010). Although, most commonly used esterification agent is vinyl-acetate, other acyl-donors can be applied efficiently as well (Li et al. 2013; Paiva et al. 2011; Teixeira and Lourenço 2014; Sahin et al. 2012). Although vinyl esters have the advantage to shift the equilibrium towards ester formation, due to the oxo-enol tautomerisation of the coproduct formed (Wang et al. 1988), vinyl acetate is a highly volatile non-renewable. Considering the environmental impact of the enantioseparation of secondary alcohols, a non-volatile and non-toxic reagent has several advantages over vinyl acetate. The physical properties of triglycerols make them applicable not only to be used as ester donor in the transesterification, but as solvent of the reactions, allowing to omit the organic solvents. Dlugy and Wolfson (Dlugy and Wolfson 2007) investigated the kinetic resolution of racemic secondary alcohols, such as 1-phenylethanol, in glycerol triacetate. High conversion (nearly

50%) and enantiomer excess (97%) were obtained in test tubes and product separation was performed by extraction with diethyl ether. However, neither the optimum reaction conditions and the kinetic parameters, nor kinetic reaction modelling approach were investigated.

In this study Novozyme 435 catalyzed kinetic resolutions of racemic 1-phenylethanol with triacetin and tributyrin were investigated. Optimization of the reaction conditions and modelling of the reaction kinetics in neat conditions agent were in the centre of the investigation. This model of the reaction kinetics could support further research in kinetic resolution of secondary alcohols and the scale up of neat reactions.

2. METHODS

2.1 Chemicals

(R,S)-1-Phenylethanol (PE: ≥98%), glycerol tributyrate/ tributyrin (GTB: ≥99%) and glycerol triacetate/ triacetin (GTA: ≥99%) obtained from Sigma-Aldrich (Budapest, Hungary). The water contents of the triglycerides are lower than 1%. Reactions were performed in the presence of *Candida antarctica* lipase B enzyme (CALB-Novozym 435, ≥ 5000 U/g) which was purchased from Sigma-Aldrich (Budapest, Hungary).

2.2 Enzymatic reactions

The kinetic resolution of racemic 1-phenylethanol catalysed by CALB was performed in the esterification agent as reaction medium. The reaction scheme is shown in Fig. 1. Experiments were carried out in capped bottles, which were placed in a tempered water bath. The temperature of the water bath was controlled in the range of 30-70°C. Liquid substrate mixture was homogenized by a magnetic stirrer set to 500 rpm. Experiments were performed accordingly: 1 ml of triacetin or tributyrin, 10-200 μl of (R,S)-1-phenylethanol and 10.0 mg of immobilized enzyme were added into the capped vials. Vials were immediately closed and placed into the water bath. Glasses were only opened for short periods required for taking samples. To follow the time course of the yield five samples were taken with a frequency of

30 min in the first two hours, then the frequency of sampling was increased to 1 hour. The volume of the samples were 5 µl each. The samples were analysed by gas chromatography (GC).

Fig. 1.

2.3 Analysis

Enantiomeric excess (*ee*) and conversion (*X*) were determined by GC analysis. GC analysis were performed on Finnigan Trace GC Ultra, using a chiral capillary column (Agilent J&W GC Columns HP-CHIRAL-20 B, 30 m × 0.25 mm × 0.25 µm). The GC was equipped with a FID detector, helium was applied as a carrier gas. The detector and injector temperatures were both set to 250°C, the head pressure was 170 kPa. Two different temperature programs were applied in the analysis of the reaction samples with triacetin and tributyrin.

For the reaction mixtures with triacetin the temperature program that initialized at 110°C for 6 minutes and continued with a temperature increase at the rate 4.5°C/min up to 175°C, kept for 3 minutes, then heated with the rate 12°C/min up to 230°C kept for 1 minute was used. Retention times: α-methylbenzyl acetate: t_R (*S*)=9.6 min, t_R (*R*) = 9.9 min; 1-phenylethanol: t_R (*R*) = 10.0 min, t_R (*S*)=10.3 min.

For the reaction mixtures with tributyrin the temperature program initiated at 110°C for 18 minutes followed by a 20°C/min heating rate to 240°C kept for 5 minutes. Retention times: 1-phenylethanol: t_R (*R*) = 12.3 min, t_R (*S*) = 13.1 min; α-methylbenzyl butyrate: t_R (*R*) = 20.9 min, t_R (*S*) = 21.0 min.

2.4   Calculations

Enantiomeric excess were calculated according to the following equation:

$$ee = \frac{c^*-c}{c^*+c} \qquad (1)$$

where *c** and *c* are the concentrations of the major and minor enantiomer, respectively.

Conversion was calculated applying the following equation:

$$X = \frac{c_{A0} - c_A}{c_{A0}} \cdot 100\% \qquad (2)$$

where c_A and c_{A0} are the actual and initial concentrations of racemic 1-phenylethanol, respectively. The enantiomers of *(R,S)*-1-phenylethanol and *(R,S)*-1-phenylethyl-acetate were baseline separated in the GC chromatograms. In the case of α-methylbenzyl butyrate, the peaks of the two enantiomeric products were not baseline separated. However, the absence of the baseline separation could cause only 2% of error in the calculations of enantiomeric excess of the product. The peak areas are directly proportional with the concentrations of the alcoholic enantiomers, while for the caluclation of product concentrations the response factors were determined (eq. 4). These criteria enable to replace the concentrations with the peak areas in Eq. 1 and Eq. 2.

Eq. 3 gives the definition of yield, determined by gas chromatography, based on calibration:

$$Y = \frac{c_{RP}}{0.5 \cdot c_{A0}} = \frac{c_{RP}}{c_{R0}} \qquad (3)$$

where c_{RP} is the concentration of *R*-product (ester) and c_{R0} is the initial concentration of *R*-alcohol. In the case of α-methylbenzyl butyrate, the absence of the baseline separation of enantiomer peaks may cause at around 1% uncertainity in the determination of product concentration.

In Eq. 3, the concentrations can be replaced with the peak areas taking into consideration the molar response factor (*β*). This factor defines a ratio of the peak areas of the alcohol and the product (ester) at the same concentrations. The calculation of the molar response factor is based on the following equation:

$$\beta = \frac{\frac{c_P}{c_A}}{\frac{a_P}{a_A}} \qquad (4)$$

where c_A and c_P are the concentrations of the alcohol and the product (ester), while a_A and a_P are the peak areas of alcohol and ester, respectively. According to the gas chromatography

based calibration, values of the molar response factors for the α-methylbenzyl acetate and the α-methylbenzyl butyrate were 0.685 and 0.718, respectively.

2.5 Kinetics

The lipase catalysed kinetic resolution – which is basically an enantioselective esterification reaction – follows the ping-pong bi-bi kinetics. The initial rate of reaction can be expressed by Eq. 5.

$$r = r_{max} \cdot \frac{c_A \cdot c_B}{K_{MB} \cdot c_A + K_{MA} \cdot c_B + c_A \cdot c_B} \qquad (5)$$

where r_{max} is the maximum reaction rate, K_{MA} and K_{MB} are the Michaelis-constants of the substrates A and B, and c_A and c_B are the concentrations of the secondary alcohol and esterification agent, respectively (Ghanem 2007).

By adding the ester-donor in large excess into the reaction mixture, the variation of the concentration of esterification agent will be negligible in the reaction phase and hence the only limiting factor of the reaction will be the secondary alcohol. Therefore Eq. 5 can be simplified to the Michaelis-Menten equation:

$$r = r_{max} \cdot \frac{c_A}{K_M + c_A} \qquad (6)$$

Due to the negligible amounts of (S)-esters in the samples in both studied systems, the initial reaction rate (r_0) can be considered as the rate of (R)-1-phenylethyl-ester production. Therefore the initial reaction rate (r_0) is the rate of (R)-1-phenylethyl-ester production.

In order to determine the initial rate of the reaction, some further assumptions were made: the studied reactions performed in a perfectly stirred, isothermal batch reactor in a constant volume reaction mixture. Although enzyme-catalysed reactions are in principle equilibrium driven, first-order reaction kinetics can be used at least at the beginning of the reaction. The kinetic equations for the (R)-enantiomer could be therefor expressed by Eq. 7:

$$-\frac{dc_{RA}}{dt} = m \cdot r(t) = m \cdot k \cdot c_{RA}(t) \qquad (7)$$

where c_{RA} is the concentration of (R)-alcohol, m is the mass of enzyme preparation, t is the reaction time, k is the reaction rate coefficient (relevant to the enzyme mass).

By combining Eq. 7 with Eq. 3 and assuming no by-product formation, excess of one reagent (trygliceride) to forward the reaction and absence of water to avoid to occur the reverse reaction (hydrolysis of ester formed), the following expression of the yield can be derived:

$$Y(t) = 1 - e^{-k \cdot m \cdot t} \qquad (8)$$

which was fitted on the measured data by Mathematica software. The reaction rate coefficient obtained by fitting of the Eq. 8 was used to determine the initial rate of reaction at $t=0$ by Eq. 7. Kinetic parameters, K_M and r_{max} were determined by fitting the Michaelis-Menten equation (Eq. 6) on the corresponding r_0 and c_{R0} values.

3. RESULTS AND DISCUSSION

3.1 Effects of the esterification agents

Esterification reactions with both ester donors were investigated at 40°C at atmospheric pressure yielded up to 92-98% of the R-ester product in 6 hours (Fig. 2). The *ee* values of the alcohol after 6 hours of reaction time were 93% and 98% applying glycerol triacetate and glycerol tributyrate as esterification agent, respectively.

Fig. 2.

The concentration of racemic secondary alcohol (c_{A0}) was varied between the concentration range of 82.7 – 1653 mM with a fixed amount of glycerol derivative (1 mL). The initial reaction rates relative to the mass of enzyme preparation are plotted against the initial R-alcohol concentration (c_{R0}) in Fig. 3. Initial rate of reaction is higher in the reactions of glycerol tributyrate as ester donor than of glycerol triacetate at any initial alcohol concentrations (Fig. 3). This phenomenon could be explained by the substrate specifity of the applied CALB enzyme. Impact of vinyl ester alkyl chain length on the catalytic efficiency and selectivity of CALB have been already reported (de los Ríos et al. 2008; Paljevac et al. 2009; Paiva et al.

2011). In terms of initial reaction rate they found that the CALB prefer vinyl butyrate. The result indicate that steric effects play important role on the catalytic activity of CALB, the initial reaction rate is effected by the distance between the alcohol and the acid binding site of the enzyme.

Fig. 3.

Maximum initial reaction rate and Michaelis constant values (Table 1) were determined by fitting Eq. 6 (Fig 3.). According to the kinetic parameters, the lipase showed higher affinity to the secondary alcohol and converted it to product more rapidly when glycerol tributyrate was applied as esterification reagent. Therefore further experiments were conducted with glycerol tributyrate as ester-donor.

Table 1.

3.2 Effect of the temperature

The kinetic resolution proved to be effective at all temperatures between 30-70 °C regarding the enantiomer separation. At any given substrate concentration (in the concentration range of 41.3 – 827 mM reagarding the selectively converted (R)-1-phenylethanol) the initial rate of the reaction is increasing with the reaction temperature until 60°C (Fig. 4). Temperature dependence of the kinetic parameters r_{max} and K_M are shown in Fig. 5

Fig. 4.

Fig. 5.

The interpretation of increasing reaction rate until 60°C can be explained by Arrhenius law like in the case of any chemical reaction. Temperature dependence of k, the reaction rate coefficient relative to the mass of enzyme preparation, could be expressed by Arrhenius equation accordingly:

$$k = A \cdot e^{\frac{-E^{\#}}{R \cdot T}} \qquad (9)$$

where A is a constant, R is the universal gas constant, T is the absolute temperature, $E^\#$ is the activation energy of the ester formation. At $c_{R0} \gg K_M$ enzyme catalysed reactions turn to be zero order reaction. Thus:

$$r_{max} = k(T) = A \cdot e^{\frac{-E^\#}{R \cdot T}} \qquad (10)$$

Activation energy could be obtained by linearization of Eq. 10 in the following way:

$$\ln r_{max} = lnA - \frac{\cdot E^\#}{R} \cdot \frac{1}{T} \qquad (11)$$

Activation energy was calculated as 30985.4 J/mol using the slope of the linearized curve (Fig. 6.) by neglecting the data measured at 70°C.

Fig. 6.

The decrease of maximal reaction rate above 60°C may be caused by the inactivation of the enzyme by heat. It becomes predominant factor instead of the Arrhenius equation at higher temperatures. The optimum temperature of CALB was found in our experiments at 60°C, which is relatively low compared to the optimal temperatures of Novozym 435 in other solvent-free systems in the literature. Yasmin et al. and Wolfson et al. studied the effects of temperature on the reaction in the temperature range of 30 – 70°C (Yasmin et al. 2006) and 25 – 80°C (Wolfson et al. 2011), respectively. The conversion increased continuously with increasing temperature at a fixed reaction time. Moreover, in case of the transestherification of isoamyl alcohol, the conversion increased linearly with the increasing reaction temperature in the range of 40 – 120 °C (Wolfson et al. 2010).

K_M is a dissociation constant of the enzyme-substrate complex to free enzyme and substrate in the Michaelis-Menten model. Therefore the temperature dependence of K_M could be written in a similar form (Sizer 1943) shown in eq. 12.

$$K_M = B \cdot e^{\frac{-\Delta G}{R \cdot T}} \qquad (12)$$

where ΔG is the free enthalpy difference of the formation of enzyme-substrate complex and B is constant. Similar linearization method as mentioned above could be used to calculate the ΔG:

$$lnK_M = lnB - \frac{\Delta G}{R} \cdot \frac{1}{T} \qquad (13)$$

ΔG is 15220.4 J/mol determined by plotting lnK_M - $1/T$, as shown in Fig. 7.

Fig. 7.

As seen in Fig. 5, K_M increases with temperature. Therefore lipase affinity for the R-alcohol is decreasing with increasing temperature.

3.3 Verification of the model

The model equations presented in section above has been applied to determine the conversion profile of a reaction ($X(t)$) from the initial concentration of the R-enantiomer (c_{R0}), the amount of enzyme (m) and the temperature of the reaction (T) by using the determined values of relevant constants. Namely with Eq. 10 and 12 the value of r_{max} and K_M were calculated for the selected temperature. By Eq. 6 r_0 was calculated followed by determination of the time dependence of R-alcohol concentration by Eq. 7, followed by calculation of $X(t)$ by Eq. 2.

The values predicted by the model are compared in Fig. 8 with the conversion values of three repeated experiments at 50°C. The average deviation of predicted from the measured values are found to be below 8%.

The model can predict the conversion profile of reactions at the temperature range in 30-60°C with acceptable accuracy, while due to the inactivation of the enzyme preparation at higher temperatures eq 12 cannot be used above 60 °C in this reaction.

Fig. 8.

4. CONCLUSIONS

Candida antarctica lipase B enzyme (Novozyme 435) catalysed kinetic resolution of racemic 1-phenylethanol was successfully performed using triacetin and tributyrin as solvents and

esterification agents. Nearly full conversions and excellent enantiomeric excesses were achieved. While CALB catalyses reactions by the ping-pong bee-bee mechanism, we've proved that the neat reaction with tributyrin can be described by the simple Michaelis-Menten kinetics. Furthermore, while it is well known, that catalytic activity and often selecitity of enzymes are strongly influenced by temperature, dependence of Michaelis-Menten constants on the temperature is rarely even mentioned. We showed, that incorporating the Arrhenius and Sitzer equations into the Michaelis-Menten model, it is suitable to describe the temperature dependence of Michaelis-Menten constants, and activation energy of 30985.4 J/mol and free enthalpy difference of 15220 J/mol were determined from the experimental data. Using this model the time frame, the concentration profile over time and the time requirement to achive a given yield can be calculated with good accuracy at any temperatures between 30-60°.

Acknowledgement

NOMENCLATURE

A	preexponential constant of the Arrhenius equation (-)
a	peak area on chromatograms (-)
CALB	*Candida antarctica* lipase B (Novozym 435)
c	concentration (mol/dm^3)
$E^{\#}$	activation energy (J/mol)
ee	enantiomeric excess (%)
GTA	glycerol triacetate
GTB	glycerol tributyrate
k	reaction rate coefficient (min^{-1}·mg enzyme^{-1})

K_M	Michaelis-constant (mol/dm^3)
m	mass of enzyme preparation (mg)
PE	1-phenylethanol
R	universal gas constant (J·mol^{-1}·K^{-1})
r	reaction rate (mol·L^{-1}·min^{-1}·mg immobilized enzyme^{-1})
T	temperature (K)
t	time (min)
X	conversion (%)
Y	yield (-)
β	molar response factor (-)
ΔG	free enthalpy difference (J/mol)
A	alcohol
B	esterification agent
m	minor enantiomer
max	maximum
P	product
RP	R-product
*	major enantiomer

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Table

Esterification agent	K_M (mol/dm^3)	r_{max} ($\cdot 10^{-4}$ mol/(dm$^3 \cdot$min$\cdot$mg enzyme))
Glycerol triacetate	0.489 ± 0.029	3.24 ± 0.09
Glycerol tributyrate	0.224 ± 0.014	7.69 ± 0.23

Table 1. Kinetic parameters at 40°C. Measurement uncertainty values are calculated accordin to the results of 3 repeated measurements.

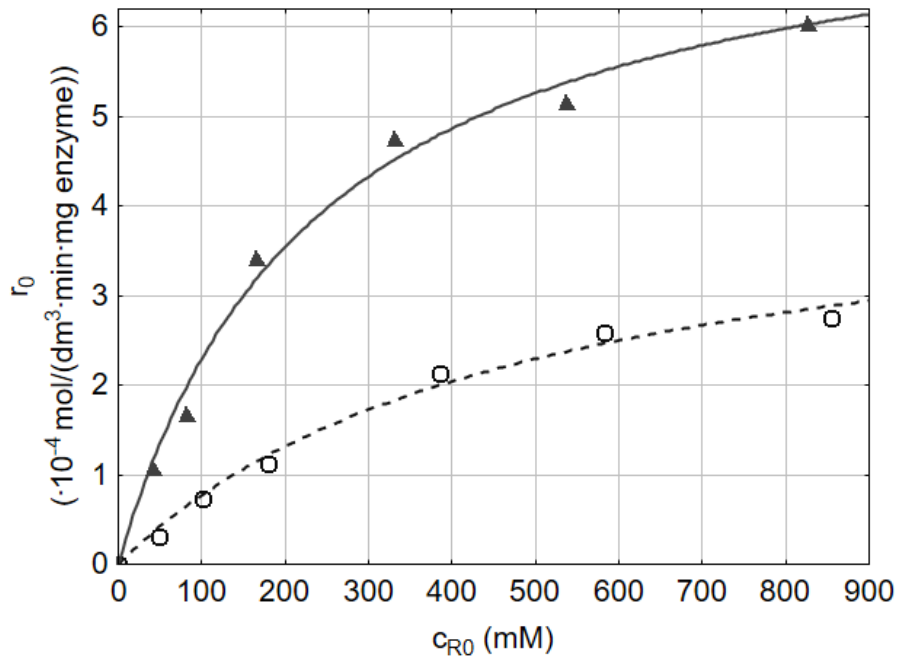**Figures**

Fig. 1.

A

B
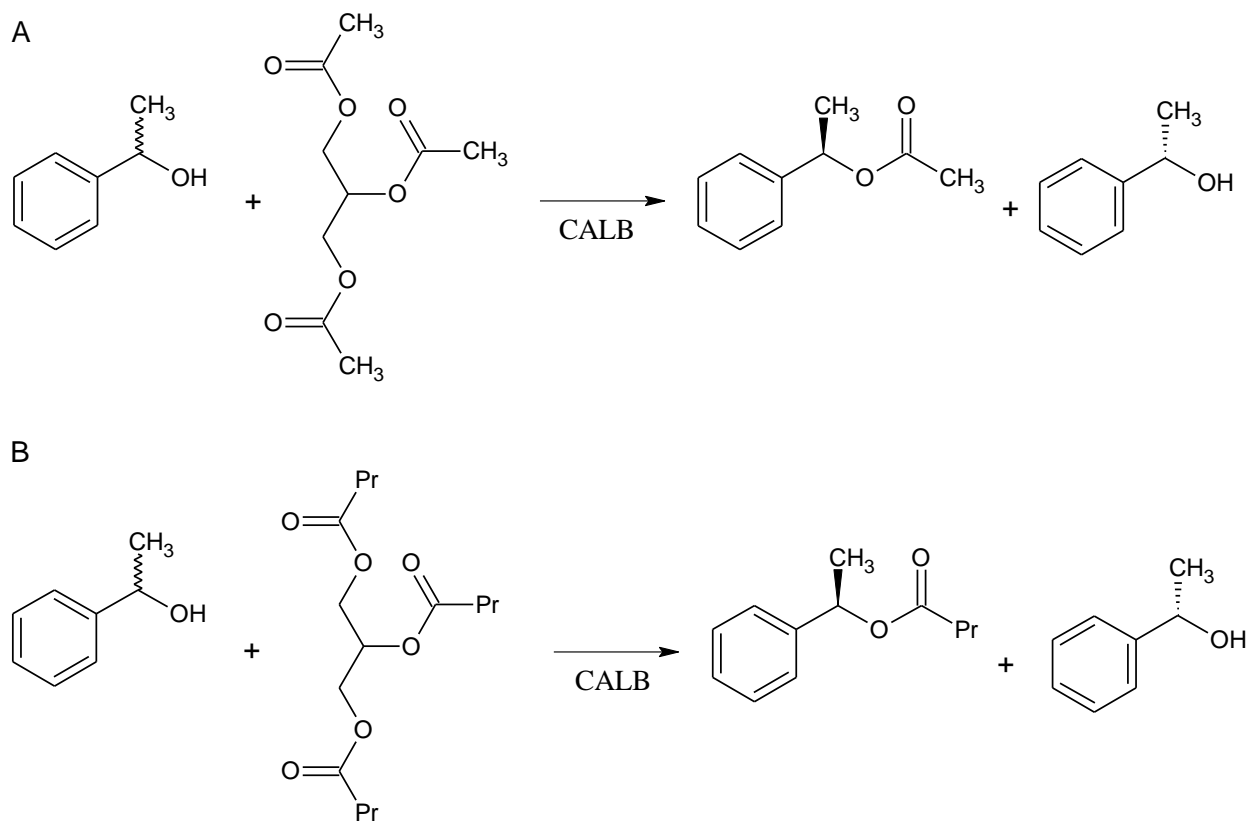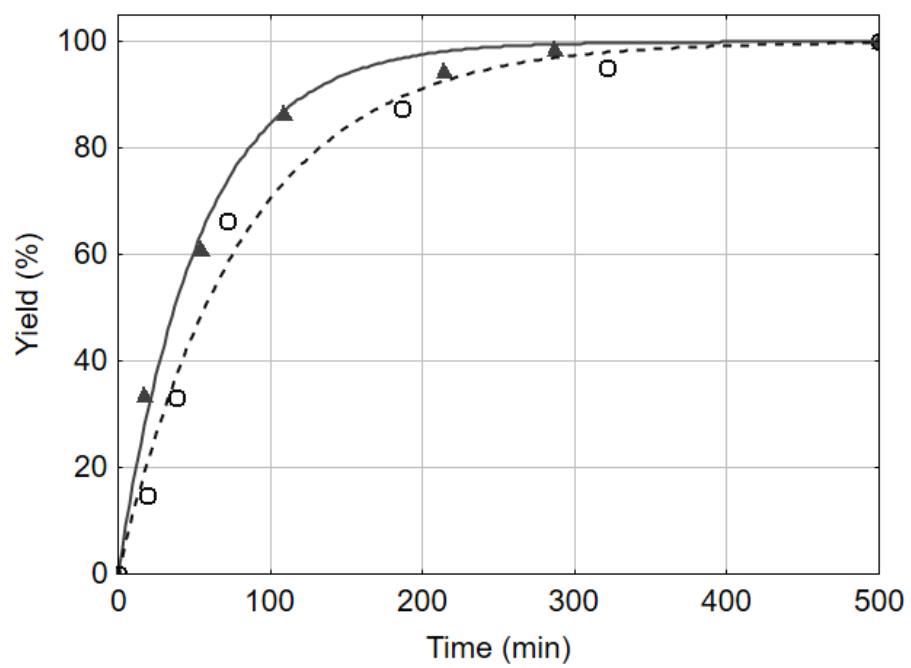
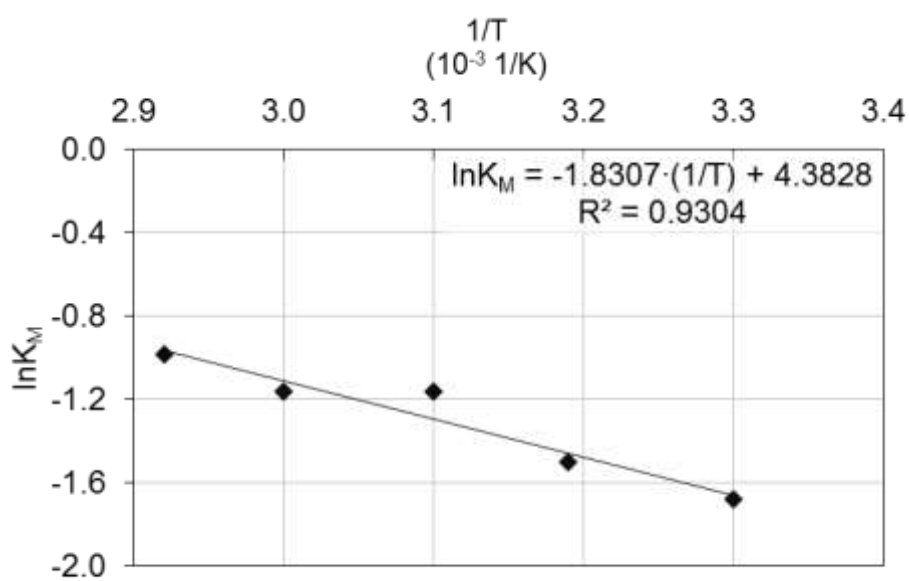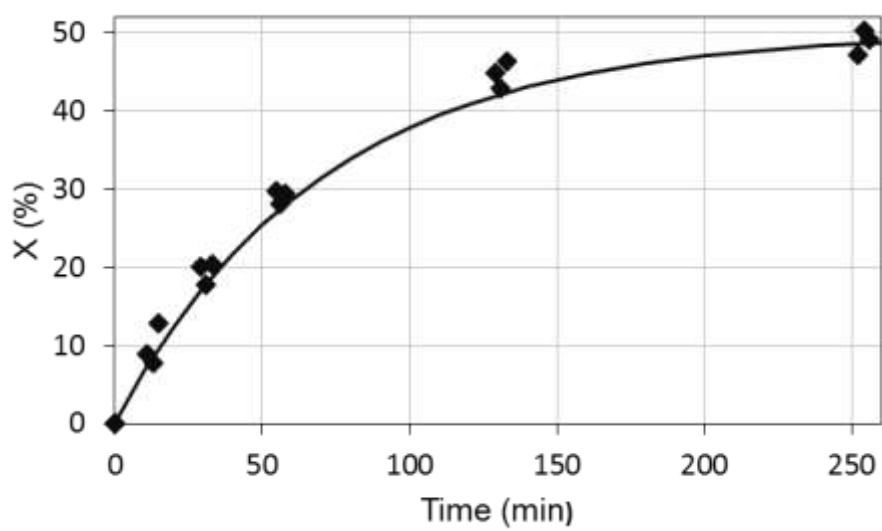**Fig.2.**

Fig. 3.

Fig. 4.
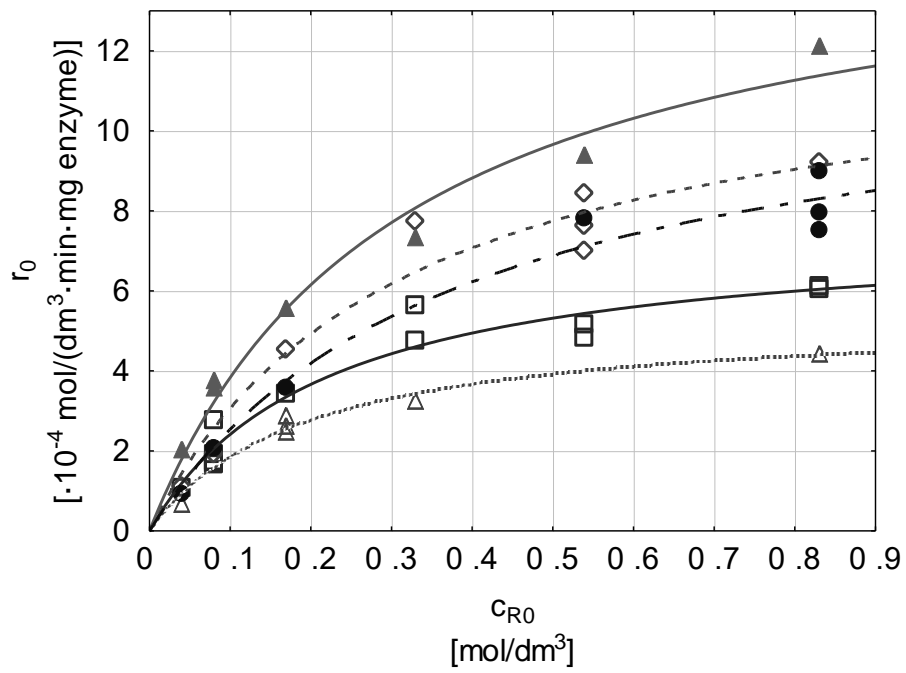
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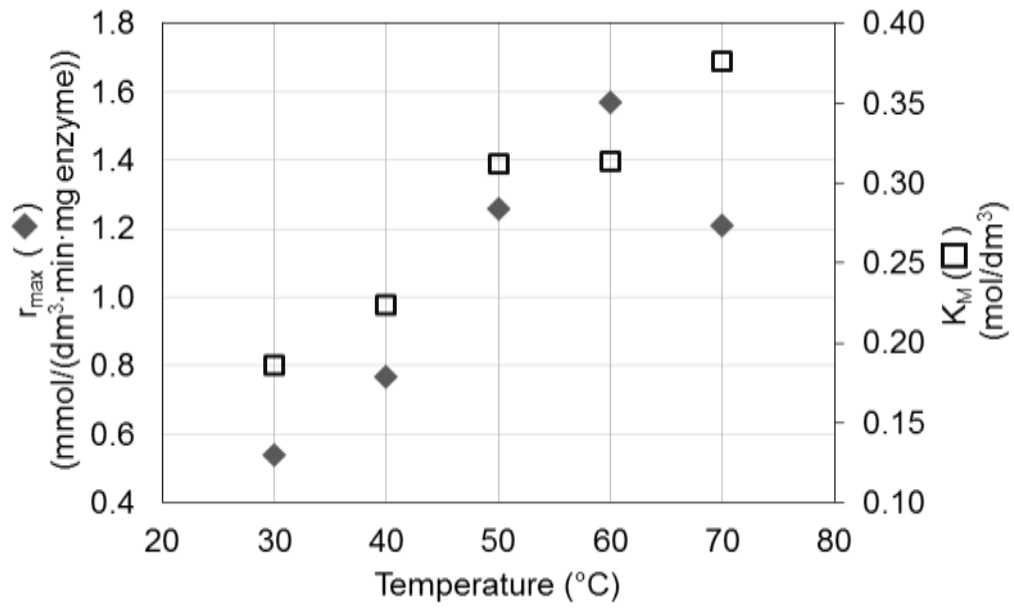
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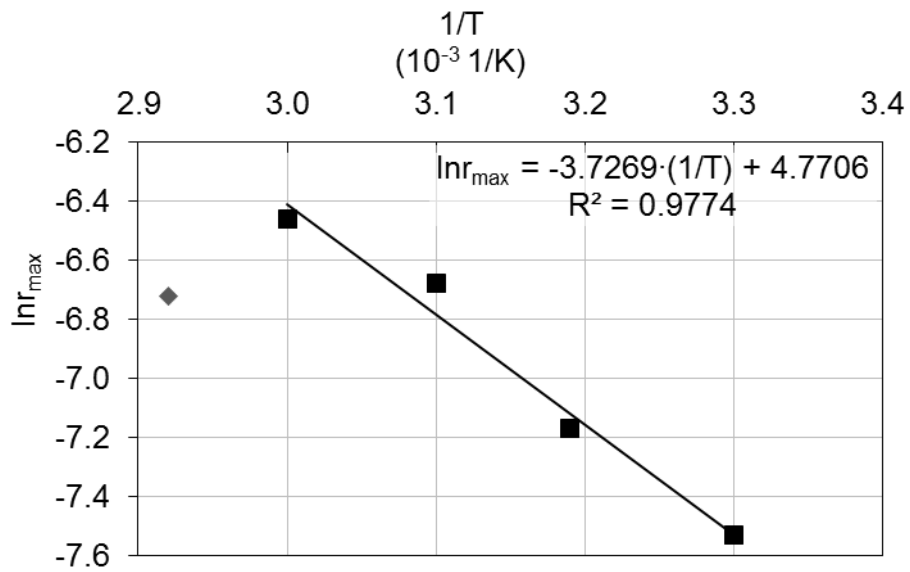
Fig.7.

Fig. 8.

Figure captions

Fig. 1. Scheme of the esterification of racemic 1-phenylethanol in neat glycerol triacetate (A) and in neat glycerol tributyrate (B)

Fig. 2 Effect of the reagent on the yield vs time, T = 40°C, initial concentration of (*R*)-1-phenylethanol 4.14·10^{-2} mol/dm^3, 10 mg enzyme preparation. Curves are fitted according to Eq.8. ▲: glycerol tributyrate ○ glycerol triacetate

Fig. 3 Effect of the applied esterification agent on the initial rate of reaction (r_0). T= 40°C, initial concentration of (*R*)-1-phenylethanol (c_{R0}) varied in the concentration range of 41.3-827 mM applying 10 mg of CALB. Fitted curves are according to Eq.6. ▲: glycerol tributyrate ○ glycerol triacetate

Fig. 4 Initial rate of reaction (r_0) versus initial concentration of (*R*)-1-phenylethanol (c_{R0}) at different temperatures. Fitted curves according to Eq. 6. ▲: 60°C ◊: 50°C ●: 70°C □: 40°C Δ: 30°C

Fig. 5. Obtained kinetic constants at the temperature range of 30-70°C. ♦: r_{max} □: K_M

Fig. 6. Linearization of the maximum initial reaction rate – temperature dependence according to Eq.11.

Fig. 7. Linearization of Michaelis constant–temperature dependence according to Eq.13.

Fig. 8 Comparison between the developed model and the experimental conversion data. ♦: measured data — : model