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Immobilization of lipase from *Thermomyces lanuginosus* and enzyme-catalyzed enantioselective alcoholysis of vinyl acetate with 2-ethyl-1-hexanol

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Catalytic properties of lipase isolated from filamentous fungus *Thermomyces lanuginosus* were investigated in this work. The possibility of enzymatic resolution of (±)-2-ethyl-1-hexanol was shown. Optimal conditions for vinyl acetate alcoholysis with 2-ethyl-1-hexanol were as follows: 0% of solvent, 0.06 g/ml *T. lanuginosus* lipase, 40 °C. *T. lanuginosus* lipase was covalently immobilized on three supports: chitosan, aminosilochrom C-80 and polyurethane. The highest immobilization yield was obtained using chitosan as a support.

Key words: lipase, immobilization, chitosan, enantioselective alcoholysis, 2-ethyl-1-hexanol

INTRODUCTION

Lipases comprise a group of enzymes that catalyze the hydrolysis of triacylglycerols in aqueous media and the reverse esterification reaction in organic solvents. Lipases, although classified as triacylglycerol hydrolases (EC 3.1.1.3), have currently been employed more frequently as powerful tools for selective transformation of unnatural substrates than as mere catalysts for splitting fats and oils [11]. Lipases as catalysts of synthesis reactions (esterification, trans- and interesterification, amidation, etc.) have numerous applications: flavors, emulsifiers, stabilizing and conditioning molecules, fatty esters of polyols as drug carriers, surfactants in the detergent and cosmetic industries, and efficient processes for enantiomerically pure products in the pharmaceutical industry [10]. Lipases are able to catalyze enantio- and regioselective as well as asymmetric reactions, involving a very wide range of key intermediates in organic and pharmaceutical chemistry (chiral alcohols, carboxylic acids, amines and so on).

To use enzymes in organic solvents, it is necessary to avoid deactivation or denaturation of protein structures, and several approaches have been formulated such as direct dispersion of powdered enzymes in organic solvents [7], the surface modified enzyme with poly(ethylene glycol) (PEG) which solubilizes the enzyme in organic solvents [6] and a lipid-coated enzyme system [8]. Following the trend in applied biocatalysis towards solvent-free processes, now the aim is usually to carry out the bioreactions without organic solvent (in solvent-free media composed only of the mixture of substrates) [1]. The aim of this article is to propose the method for enzymatic resolution of (±)-2-ethyl-1-hexanol in a solvent-free system.

The binding of lipases on suitable pre-existing supports should greatly improve the performance of industrial reactors allowing us a continuous use or re-use of such interesting biocatalysts. Because of a wide variety of the reaction systems in which lipases can be used, the preparation of immobilized derivatives has to be done according to very different points of view. Immobilization of lipases could be also used as a tool to improve and optimize some activity/stability parameters [5]. In this work, *Thermomyces lanuginosus* lipase was covalently immobilized on three supports: chitosan, aminosilochrom C-80 and polyurethane.

EXPERIMENTAL

Materials

Lipase isolated from the filamentous fungus *Thermomyces lanuginosus* (Lipolase 100 T™) was obtained from Novozymes A/S (Bagsvaerd, Denmark). 2-octanol, 2-ethyl-1-hexanol, vinyl acetate, chitosan (2-amino-2-deoxy-(1→4)-β-glucopyranan, M. W. 750000) (Fluka), n-hexane, tributyrin, aminosilochrom C-80 (Reachim) were of reagent grade and were used as received. Bicomponent composition HYPERLAST was a product of Derbshire (UK). HYPERLAST ISO-

CYANATE 5003 contained 60–100% of diphenylmethane diisocyanate, HYPERLAST 7982016 contained 5–10% of 1,4-butanediol.

Experimental and apparatus used

Thermostat UTU-4 (Poland), pH-meter I-130 (Russia), ultrasonic desintegrator UZDN-A (Russia), analytical scale Mettler AE 200 (Switzerland), gas chromatograph Chrom 5 (Prague).

Synthesis of 2-octyl acetate

2-octyl acetate was prepared according to a modified procedure [9]. 47.3 ml (0.5 mol) of distilled acetic anhydride and 79.5 ml (0.5 mol) of distilled 2-octanol were mixed in a round-bottom flask equipped with an Allihn condenser closed with a $MgSO_4$ tube. After 5 drops of conc. sulfuric acid were added the mixture was heated at 100–110 °C for two hours. After cooling down the reaction mixture was poured out into a flask with 150 ml of freezing water. The aqueous and the organic layers were separated. The aqueous layer was extracted twice with chloroform (2 portions 50 ml each). The organic layer and chloroform extract were combined and neutralized with 1.25 M Na_2CO_3 solution. The obtained mixture was washed with distilled water (3 portions 100 ml each) and dried over anhydrous $MgSO_4$ overnight. After filtration and fractional distillation pure 2-octyl acetate was obtained.

Investigation of *Thermomyces lanuginosus* lipase synthetic activity

To a 2-ethyl-1-hexanol and vinyl acetate mixture (molar ratio 1:3) n-hexane was added so that the final concentration of n-hexane was 0%(v/v), 50%(v/v) and 75% (v/v). To the thermostated (30, 40 and 50 °C) solution enzyme powder was added under continuous stirring to give a concentration of 30.4, 45.5, 60.8 and 91.2 mg/ml. The reaction kinetics was monitored by taking 1 ml aliquots of reaction mixture. Supernatant solutions obtained after centrifugation were stored at 4 °C in vitreous test-tubes until further analysis. Reaction samples were analyzed on a Chrom 5 gas chromatograph equipped with an OV-17 column (10 meters). The carrier gas was nitrogen, the column temperature was risen from 80 °C to 230 °C at the rate 20 °C/min. The final temperature (230 °C) was kept until complete elution. The molar 2-ethyl-1-hexanol to 2-ethylhexyl acetate ratio was found from gas chromatograms. The retention times of vinyl acetate, 2-ethyl-1-hexanol and 2-ethylhexyl acetate were 3–4 min, 6–7 min, and 7–8 min respectively.

Immobilization of lipase

Covalent immobilization on C-80aminosilochrom. A published procedure [3] was used. To 10 ml of 2.5% aqueous glutaraldehyde solution 0.5g dry aminosilochrom were added and the mixture was vacuumed for 10 min under 50 mm Hg. After 20 min of stirring under atmospheric pressure, aminosilochrom was filtered off and washed with distilled water. The aminosilochrom powder was stirred for additional 20 min to remove sorbed glutaraldehyde and washed with (10 ml) 0.02 M phosphate buffer (pH 7.0). A mixture of 0.5 g wet aminosilochrom in 1 ml phosphate buffer and 1 ml lipase solution (0.5 mg/ml) were stirred for 1 hour at 37 °C. The obtained granules were washed with 2 ml of phosphate buffer.

Covalent immobilization on chitosan microspheres. A published procedure [12] was used. The enzyme was immobilized on citosan microspheres after activation and sewing with glutaraldehyde. To 30 ml of warm (50 °C) 1% chitosan solution in 2% acetic acid 19.8 ml of 0.5 M NaOH was added. After 10 min 0.5 ml of 25% glutaraldehyde solution was added dropwise. The obtained microspheres were filtered, washed with excessive water and 10 ml 0.05 M phosphate buffer (pH 7.00). The slurry of 0.5 g wet chitosan in 2 ml of phosphate buffer and 1 ml lipase solution in phosphate buffer (0.5 mg/ml) were stirred for 1 hour at 37 °C. The obtained microspheres were washed with 2 ml of phosphate buffer.

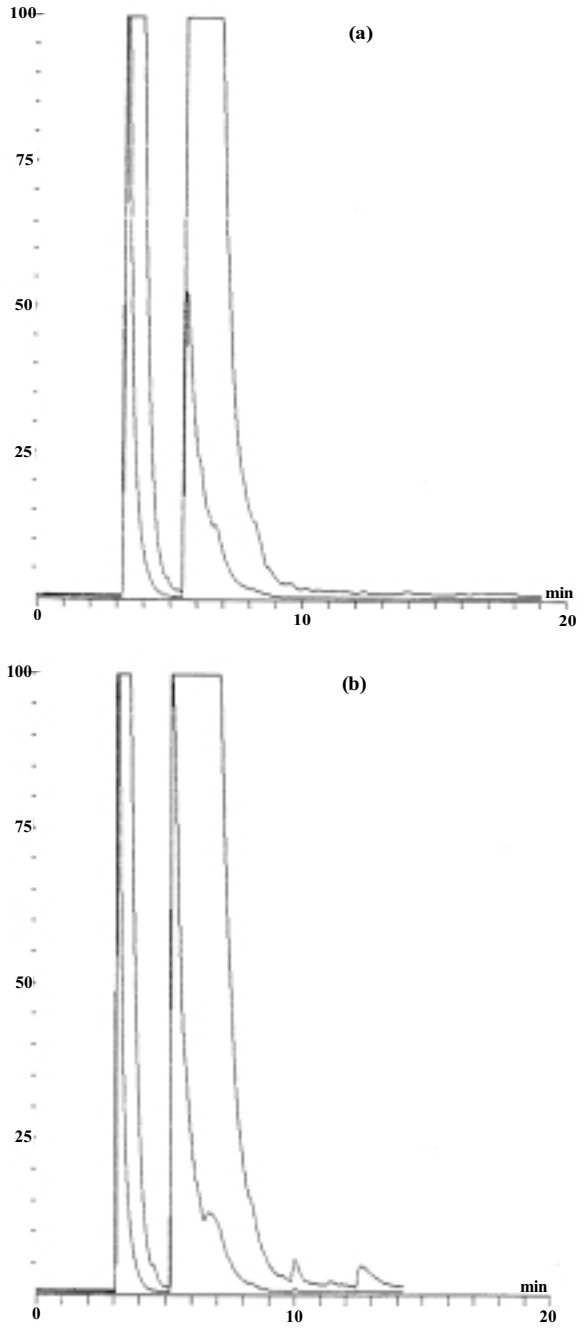
Immobilization on polyurethane. A modified procedure [2] was used. One gram of HYPERLAST 7982016, 3.5 g of HYPERLAST ISOCYANATE and 1 ml of the initial lipase solution (10 mg/ml) were stirred in a small vessel until stiffness. The obtained porous substance was cut into small pieces and stored in phosphate buffer overnight. The immobilization product was filtered off and washed with buffer.

RESULTS AND DISCUSSION

T. lanuginosus lipase-catalyzed alcoholysis of vinyl acetate with 2-ethyl-1-hexanol

For this study chiral alcohol is necessary. An attempt has been made to use a simple asymmetric alcohol, 2-octanol. However, the alcoholysis reaction of vinyl acetate with this alcohol was extremely slow (reaction conditions: 2.29 M 2-octanol, 6.89 M vinyl acetate, 91.2 mg/ml lipase, 50 °C). Chromatograms taken at the beginning of the reaction and after 6 hours showed no significant difference (Fig. 1, a and b). Moreover, a characteristic peak of 2-octyl acetate (Fig. 1 c) in a gas chromatogram taken after 6 h was undetectable (Fig. 1 b). As a result,

one can conclude that *Thermomyces lanuginosus* lipase is not specific to secondary alcohols, and a substituted primary alcohol (2-ethyl-1-hexanol) was chosen for this study. Esters are usually obtained by esterification between an alcohol and an acyl donor such as free fatty acids or simple derivatives (*e.g.*, methyl or ethyl esters). In such a case, efficient removal of by-products (water, methanol, or ethanol) must be ensured to drive the reaction to completion at reasonable reaction times. Thus, alcoholysis reaction was performed using activated ester (vinyl acetate), allowing for an irreversible and rapid synthesis.



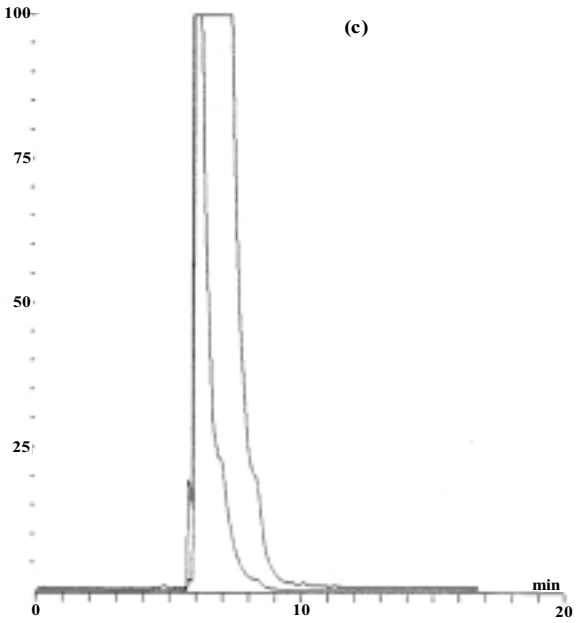


Fig. 1. Gas chromatogram of reaction mixture consisting of 2.29 M 2-octanol, 6.89 M vinyl acetate and 91.2 mg/ml lipase (*a*), same reaction mixture after 6h incubation at 50 °C (*b*), and genuine 2-octyl acetate (*c*). Carrier gas: N_2, column: OV-17, 10 m long, elution conditions: temperature gradient 80 °C to 230 °C at 20 °C/min rate, steady state at 230 °C until complete elution

After effective reactants for re-esterification reaction had been found, the reaction conditions were optimized. The amounts of reactant 2-ethyl-1-hexanol and reaction product 2-ethylhexyl acetate were quantitatively determined integrating the corresponding peaks in gas chromatograms. The amount of lipase was optimized by following the conversion of the initial 2-ethyl-1-hexanol into 2-ethylhexyl acetate (Fig. 2). The yield of the reaction product increases from 30.4 mg/ml to 60.8 mg/ml of lipase, however, the further increase from 60.8 to 91.2 mg/ml has no significant effect on the reaction yield. One can conclude that the optimal enzyme concentration is 60.8 mg/ml, and higher concentrations do not increase the conversion.

Enzyme-catalyzed reactions are known to be temperature-dependent [4], and the reaction temperature was optimized in this study. The effect of temperature on the enzymatic activity of *T. lanuginosus* lipase is shown in Fig. 3. The reaction rate at 30 °C is considerably lower than at higher temperatures (40 °C and 50 °C), which is expected for most chemical reactions. The further increase of reaction temperature from 40 °C and 50 °C increased the initial reaction rate, however, after 4–5 hours incubation conversion was higher in the reaction medium at 40 °C than that at 50 °C. Lower alcoholysis yields

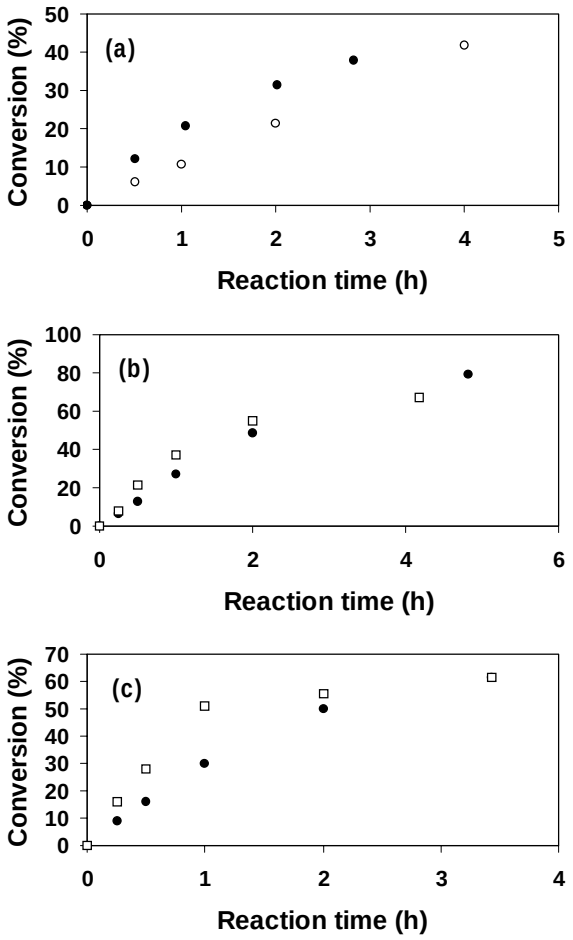


Fig. 2. Conversion (%) of 2-ethyl-1-hexanol into 2-ethylhexyl acetate. Reaction conditions: 1.17 M 2-ethyl-1-hexanol, 3.42 M vinyl acetate, n-hexane (50% v/v) at 30 °C (a), 40 °C (b) and 50 °C (c). Amount of *Thermomyces lanuginosus* lipase 30.4 mg/ml (❍), 60.8 mg/ml (●) and 91.2 mg/ml (□)

at 50 °C can be explained by enzyme inactivation. Thus, one can conclude that the optimal temperature for lipase-catalyzed alcoholysis of vinyl acetate with 2-ethyl-1-hexanol is about 40 °C.

Many reactions catalyzed by lipases proceed more effectively in the presence of organic solvent. Organic solvent provides a buffering effect in respect to substrate composition variation, water partitioning, and reduces viscosity of reaction medium [10]. However, the use of organic solvents in any process is associated with additional steps required for solvent separation and regeneration and potential negative effects due to their presence in a final product. The costs associated with solvent use often create economic barriers for the enzyme-catalyzed process. Solvent-free processes are especially attractive for industrial applications since the volumetric yield in processes based on organic solvents is lower. The effect of n-hexane on vinyl acetate alcoholysis with 2-ethyl-1-hexanol is shown in Fig. 4.

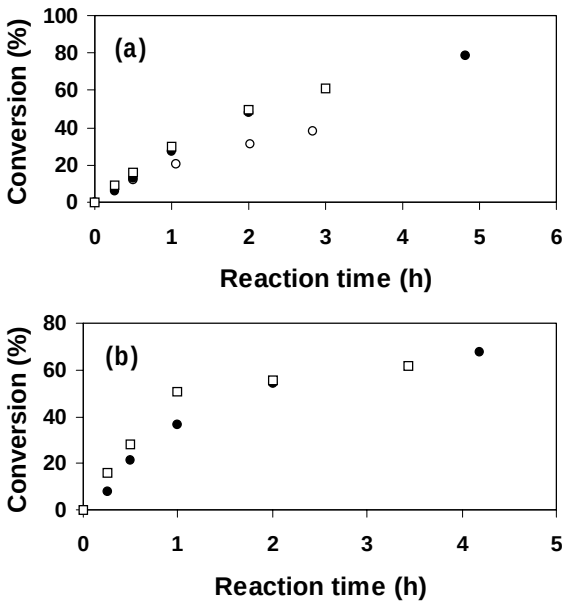


Fig. 3. Conversion (%) of 2-ethyl-1-hexanol into 2-ethylhexyl acetate. Reaction conditions: 1.17 M 2-ethyl-1-hexanol, 3.42 M vinyl acetate, n-hexane (50% v/v). Amount of *Thermomyces lanuginosus* lipase added 60.8 mg/ml (a) and 91.2 mg/ml (b). Incubation temperature 30 °C(❍), 40 °C(●) and 50 °C(□)

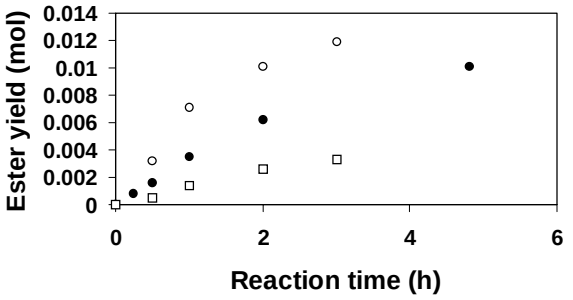


Fig. 4. Effect of n-hexane on the amount of 2-ethylhexyl acetate produced by *Thermomyces lanuginosus* lipase-catalyzed alcoholysis of 2-ethyl-1-hexanol and vinyl acetate. Reaction conditions: 2-ethyl-1-hexanol, vinyl acetate (molar ratio 1:3), 60.8 mg/ml biocatalyst, 40 °C. Amount of n-hexane added: 0% (v/v) (❍), 50%(●) and 75%(□)

These results clearly demonstrate that addition of n-hexane to the reaction mixture is not necessary since 2-ethylhexyl acetate synthesis is faster in a medium containing only reactants and the enzyme.

The possible method for enzymatic resolution of (±)-2-ethyl-1-hexanol using alcoholysis with vinyl acetate

The lipase-catalyzed enantioselective alcoholysis of vinyl acetate with 2-ethyl-1-hexanol was carried out in a solvent-free system with stirring. The reaction conditions were as follows: 2.30 M 2-ethyl-1-hexanol, 6.94 M vinyl acetate, 45.5 mg/ml biocatalyst, 40 °C.

Optical rotation for both enantiomers is equal to 0, and polarimetry cannot be used to determine the amount of each enantiomer. Therefore, the mathematical approach was employed to find the reaction rates of two optical isomers of 2-ethyl-1-hexanol. The kinetic model for analyzing the experimental data was proposed. Since one of the reactants (vinyl acetate) is always present in great excess and the concentration of enzyme is constant (lipase remains in solid state during irreversible alcoholysis reaction and variation in free enzyme concentration does not greatly affect the equations describing the enzymatic reaction), the kinetic equations for different enantiomers of 2-ethyl-1-hexanol may be written as for the first-order reactions:

(R)-2-ethyl-1-hexanol → (R)-2-ethylhexyl acetate, $c(t) = a \cdot \exp(-k_1 \cdot t)$;

(S)-2-ethyl-1-hexanol → (S)-2-ethylhexyl acetate, $c(t) = a \cdot \exp(-k_2 \cdot t)$;.

Thus, the concentration of (R/S)-2-ethyl-1-hexanol and the alcoholysis reaction rate may be expressed as the sum of two exponents.

The amount of 2-ethyl-1-hexanol during alcoholysis of vinyl acetate decreases with time (Fig. 5 A), and experimental data points can be fitted into the exponential decay curve described by the general equation $c(t) = A \cdot exp(B \cdot t) + C \cdot exp(D \cdot t) + E$, where $c(t)$ is the concentration, t is the reaction time, A, B, C, D and E are time and concentration independent constants. Experimental data points were fitted and the R-squared value was calculated using the Origin 7 scientific analysis package. After differentiating the equation of reaction rate (v) was obtained: $v = A \cdot B \cdot exp(B \cdot t) + C \cdot D \cdot exp(D \cdot t)$ with the values $A = 0.382$, $B = -13.6$, $C = 0.958$, $D = -0.327$, $E = 0.956$.

The rate of the reaction (Fig. 6) as a function of time is shown in Fig. 5 B. Here the total reaction rate v is the sum ($v = v_1 + v_2$) of the rates of two reactions for R and S enantiomers of 2-ethyl-1-hexanol. The initial rate of the slower-reacting enantiomer (u) can be obtained by extrapolating the linear part of the curve in Fig. 5 B to the zero time. Then the initial rate of the more rapidly reacting enantiomer (v) may be obtained as the difference of the total reaction rate at t = 0 and the rate of the slower reacting enantiomer (u). For the experimental data shown in Fig. 5 B, $u = 0.316$, $v = 5.18$. The relative reaction rates at t = 0 of two optical isomers of 2-ethyl-1-hexanol are: $v/(v+u) \cdot 100\% = 94\%$ (for the more rapidly reacting enantiomer)

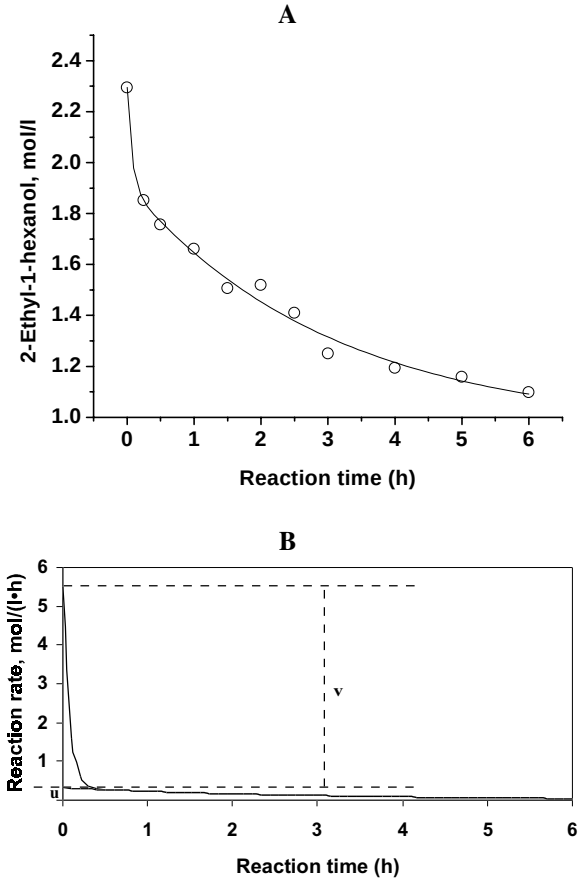


Fig. 5. (*A*) Concentration of 2-ethyl-1-hexanol as a function of time during *Thermomyces lanuginosus* lipase-catalyzed alcoholysis of vinyl acetate with 2-ethyl-1-hexanol. Points represent experimental data, solid line is exponential fit. (*B*) Reaction rate of *Thermomyces lanuginosus* lipase-catalyzed alcoholysis of vinyl acetate with 2-ethyl-1-hexanol as a function of time. Reaction conditions were as follows: 2.30 M 2-ethyl-1-hexanol, 6.94 M vinyl acetate, 45.5 mg/ml biocatalyst, 40 °C

and $u/(v+u) \cdot 100\% = 6\%$ (for the slower reacting enantiomer). The results demonstrate that *T. lanuginosus* lipase is more specific to one of 2-ethyl-1-hexanol enantiomers and that partial reso-

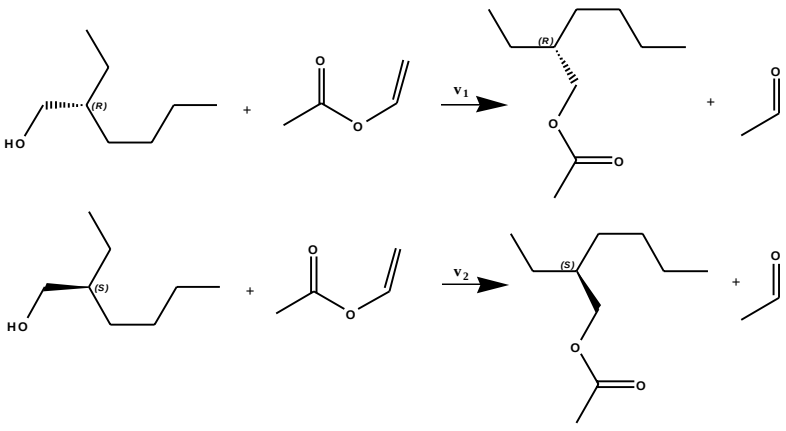


Fig. 6. Scheme of (±)-2-ethyl-1-hexanol alcoholysis with vinyl acetate

lution of the enantiomers by carrying out the reaction shown in Fig. 6 is possible.

Effect of immobilization

Immobilization is a suitable approach, which allows biocatalyst reuse, makes product recovery easier and is able to enhance resistance against inactivation by different denaturants. The preparation of immobilized derivatives of lipases may be useful to develop industrial processes of organic synthesis and is an important field of research.

T. lanuginosus lipase was covalently immobilized on three supports: chitosan, aminosilochrom C-80 and polyurethane. Chitosan (natural polysaccharide) and aminosilochrom (porous SiO_2) have a large number of available amino groups that react with glutaraldehyde. Another aldehyde group reacts with the amino group of the enzyme molecule. In this a way the enzyme is bound on the support. Diphenylmethane diisocyanate reacts with 1,4-butanediol to give polyurethane. This polymer contains active isocyane (-N = C = O) groups that effectively bind to the active enzyme groups (-OH, $-NH_2$). The activity of the immobilized biocatalyst was measured in the hydrolysis reaction of tributyrin. The reaction conditions were as follows: 20 ml of tributyrin emulsion (0.05 M NaCl, 0.5 mM KH_2PO_4, 1.23 M glycerol, 0.996 mg/ml gum arabic, 0.174 M (5% v/v) tributyrin), 0.1 g of lipase immobilized on chitosan and aminosilochrom, or 1.5 g of lipase immobilized on polyurethane; pH 7.00; 30 °C. The immobilization yield is defined as the ratio of native lipase activity and the activity of immobilized preparation loading the same amount of enzyme.

A significantly higher immobilization yield (60%) was obtained using chitosan as a support. It can be explained by a specific access of the substrate to the immobilized biocatalyst and/or a steric rearrangement of immobilized lipase. The beneficial effect of immobilization on the active site is also quite possible. The immobilization yield using aminosilochrom and polyurethane as a carrier was 38% and 13%, respectively.

Enzymatic hydrolysis of triacylglycerols and esters

The lipolytic activity of *Thermomyces lanuginosus* lipase was determined titrimetrically using the convenient pH-stat method (data not shown). The hydrolytic activity of *Thermomyces lanuginosus* lipase in respect to various triglycerides was found. The rate of hydrolysis decreases in the row: tributyrin > triolein > natural oils (olive oil, cod-liver oil, castor oil, palm oil, rapeseed oil, sunflower oil). *T. lanuginosus* lipase hydrolyzes only one of three ester bonds in the triglyceride molecule. *T. lanuginosus* lipase hydrolyzes esters of long chain primary alcohols more rapidly than shorter ones.

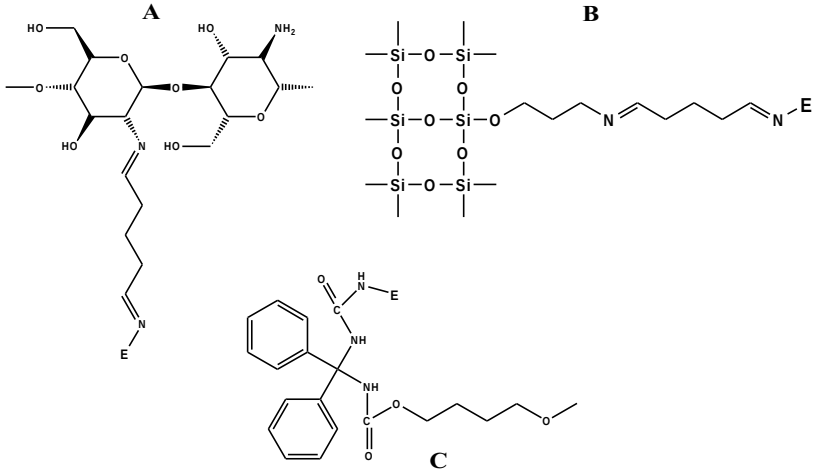


Fig. 7. Scheme of covalent immobilization products. Carrier: chitosan (*A*), aminosilochrom C-80 (*B*) and polyurethane (*C*). Letter E stands for enzyme molecule

CONCLUSIONS

1. The optimal conditions for the lipase-catalyzed alcoholysis of vinyl acetate with 2-ethyl-1-hexanol were found. They are as follows: 0% of solvent, 0.06 g/ml *T. lanuginosus* lipase, 40 °C. Lipase is more specific to one of the 2-ethyl-1-hexanol enantiomers.

2. Vinyl acetate reaction with 2-octanol was not catalyzed by lipase, and we can conclude that *T. lanuginosus* lipase is specific to primary alcohols.

3. Enzyme activity after immobilization on various supports was determined, and the highest immobilization yield was obtained using chitosan.

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***THERMOMYCES LANUGINOSUS* LIPAZĖS IMOBILIZAVIMAS IR ENANTIOSELEKTYVI LIPAZĖS KATALIZUOJAMA VINILACETATO ALKOHOLIZĖS REAKCIJA SU 2-ETILHEKSANOLIU-1**

S a n t r a u k a

Katalizuojant lipazei iš *Thermomyces lanuginosus* vyksta vinilacetato alkoholizės reakcija su 2-etilheksanoliu-1. Optimaliausios reakcijos sąlygos: 40°C temperatūra, 0,06 g/ml fermento, 0% tirpiklio. Fermentas specifiškas vienam iš 2-etilheksanolio-1 enantiomerų (alkoholio optinių izomerų santykis reakcijos pradžioje: 94% (vieno antipodo), 6% (kito antipodo)). Vinilacetato alkoholizės reakcijoje nedalyvauja 2-oktanolis, t. y. lipazė iš *Thermomyces lanuginosus* specifiška pirminiams alkoholiams.

Thermomyces lanuginosus lipazė imobilizuota ant trijų nešiklių: chitozano, aminosilochromo C-80 ir poliuretano. Didžiausia imobilizavimo išeiga gauta panaudojus nešiklį chitozaną.