VILNIUS UNIVERSITY

DOVILĖ ŠINKŪNIENĖ

LIPASE SELECTION AND APPLICATION FOR FATTY ACID ESTER SYNTHESIS

Summary of doctoral dissertation
Physical sciences, Biochemistry (04 P)

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The research has been carried out during 2008–2013 in Vilnius University, Department of Biochemistry and Molecular Biology (Vilnius, Lithuania) and Lund University, Department of Biotechnology 2013 01–04.

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Contents

INTRODUCTION

How can we obtain a good reaction yield? It is a question that continuously bothers both scientists and industrialists, regardless of the type of compounds or the means of synthesis. Biotechnologists are not the only ones to search for the answer, but it seems that it is even more difficult to find it when reactions are catalyzed by enzymes. Enzymes are products of the living organisms and sometimes are (not unreasonably) called "capricious" and "unpredictable". Indeed, we can expect a good yield and fast reaction only after "personally getting in touch" with a particular enzymatic catalyst and studying its properties and the influence of reaction conditions on the enzyme's activity and the reaction course.

Lipases (EC 3.1.1.3) are the enzymes which in nature catalyse fat splitting (triacylglycerol hydrolysis) reaction. In biotechnology they are usually and widely used not only for hydrolysis, but also for synthesis reactions, where the target products are esters and other compounds. One of the most important nowadays products are fatty acid short-chain alcohol esters (biodiesel). The extensive research on the topic is being carried out in order to apply enzymatic catalysis on industrial scale and reduce the more expensive and non-renewable fossil fuel usage. Equally important is the research and application of lipases for the synthesis of other esters, which have important properties such as flavour, fragrance, or complex structure regioisomers, enantiomers – the precursors for fine chemical or drug synthesis and for enantiomeric resolution, also for lipophilisation reactions. Lipase attractiveness and popularity lies behind their properties to catalyze hydrolysis, interesterification (alcoholysis, acidolysis), esterification, aminolysis reactions in mild conditions, using labile substrates (for example those with unsaturated bonds) and, what is especially important, high tolerance for organic solvents, high selectivity and resulting high product purity.

Successful catalysis depends on many factors – substrate and enzyme selection, enzyme preparation, reaction conditions and other. We have studied the influence of enzyme preparation (immobilization), enzyme and substrate selection and reaction condition influence for the important industrial reactions: fat hydrolysis, biodiesel synthesis and flavour and fragrance ester synthesis.

The main objective of this research was to study and optimize fatty acid ester synthesis catalyzed by lipases.

The research was aimed at:

1. Studying *Enterobacter aerogenes* lipase (isolated in Lithuania) immobilization ant its influence on catalytic activity.
2. Studying the hydrolytic and synthetic properties of commercial lipases for model, natural and complex substrates and optimization of reaction parameters.
3. Evaluation of reaction additives as acyl-group migration catalysts and their influence on triolein ethanolysis (biodiesel synthesis) reaction.
4. Influence of lipase specificity with respect to different acylglycerol classes and regioisomers on triolein ethanolysis.
5. Application of lipases for biodiesel synthesis based on the knowledge of their specificity to acylglycerols, influence of additives on acyl-migration and intermediate reaction product conversions.
6. Enzyme selection and phenethyl octanoate synthesis optimization.

Scientific novelty

The investigated *Enterobacter aerogenes* lipase has been recently isolated in Lithuania (JSC „Biocentras“) the lipase immobilization, determination of optimal reaction conditions and application for synthesis reaction were studied for the first time.

Linseed oil and pork fat mixture has been recently found to be an appropriate substrate for biodiesel synthesis reaction. The enzymes selected for transesterification of this substrate were investigated for the first time. Comparison of triolein hydrolysis and transesterification in hexane, by means of response surface methodology (RSM) is also unique, especially with regards to rarely described in literature industrial enzymes (Lipopan F® BG, Lipopan® 50 BG, Lecitase).

Enzymatic biodiesel synthesis is a widely studied subject; nevertheless, investigation of intermediate reaction product conversions, regioisomeric composition and enzyme application on the base of this knowledge is a new concept. Even though acyl-group migration concept is widely acknowledged and regarded as the main reason for high transesterification yields while using 1, 3-specific enzymes, the purposive acyl-

migration catalysis is rarely used, therefore our research was a step forward in this complex field.

Phenethyl octanoate synthesis was optimized for the first time and high yields were obtained using immobilized Lipozyme® RM IM for transesterification reactions.

Doctoral dissertation contents

Original doctoral dissertation (in Lithuanian) contains the following parts: Introduction, Literature review, Materials and Methods, Results and discussion, Conclusions, Reference list (268 references cited), List of publications (2 ISI WOS papers), Participation in conferences (4 events), Appendix, 84 Figures, 39 Tables; 205 pages in total.

MATERIALS AND METHODS

Lipases. *Enterobacter aerogenes* - 13 lipase was received from JSC „Biocentras“, Vilnius, *Pseudomonas mendocina* lipase was received from Biochemistry institute, Vilnius. Other lipases: commercial Novozymes® preparations were kindly donated by Novozymes representatives in Lithuania – JSC „Biopolis“. Lipases studied in Lund university were obtained from Sigma (St. Louis, MO), except of *Candida antarctica* (Lipozyme® CALB L) (CALB) donated by Novozymes (Denmark), *Pseudomonas fluorescens* (PFL), obtained from Aldrich, *Aspergillus niger* (ANL) from Fluka.

Enzyme carriers used for immobilization were macroporous polypropylene Accurel MP-1000, obtained from Membrana GmbH (Germany). Chitin (CHT) was bought from Reachim, Lignin was obtained from Latvian wood chemistry institute. Chitosan (CHZ) ant its magnetic derivatives were prepared from chitin by V. Bendikienė [1, 2]. Polyurethane (PU) and chitosan graft copolymer derivatives were prepared in Polymer chemistry department of Vilnius University [3, 4]. Granocell (GR) macroporous cellulose carriers, prepared according Lithuanian patent no. 2299, were obtained from Kaunas University of Technology [5].

Lipase immobilization. Lipases were immobilized by adsorption onto the macroporous polypropylene Accurel MP1000 (particle size 200-700 μm), using a modification of the method of Hagström et al. [6]. The following amounts were used per

gram of Accurel MP1000: CALB 3.3 ml, *Rhizopus arrhizus* lipase (RAL) 600 mg, *Rhizomucor miehei* lipase (RML) 1.4 ml and *Thermomyces lanuginosus* lipase (TLL) 1.4 ml. In all cases, the initial lipase preparation (solid or liquid) was dissolved in 10 ml sodium phosphate buffer per g support (pH 7.0, 50 mM), and added to Accurel MP1000, which was pre-wetted with ethanol (3 ml/g support). The support and the enzyme solution were incubated for 3 hours on a nutating mixer at room temperature, and then filtered. The preparation was rinsed 3 times with 5 ml sodium phosphate buffer (pH 7.0, 50 mM), filtered and dried overnight under reduced pressure. Lipase adsorption on other supports was carried out in the same manner as on MP1000, except that the carriers were pre-wetted with excess of buffer and the immobilisation volume was 2ml/g carrier. After 5 hours shaking in room temperature they were left overnight at +4ºC before filtration.

Covalent immobilization. The carriers, containing amino groups were activated by adding 25-30 molar excess of glutar aldehyde (GA). The excess GA was removed by filtrating and rinsing with pH 7 buffer. Further steps were conducted as described in adsorption method.

Protein content was determined by Bradford method [7] using bovine serum albumin for standard curve. The amount of lipase adsorbed onto Accurel MP1000 was determined by measuring the amount of protein in the solution before and after adsorption.

Lipase activity measurements. The hydrolytic activity of the lipase was measured using a spectrophotometric assay based on the hydrolysis of *p*-nitrophenyl butyrate (pNPB). A 20 mM stock solution of pNPB was prepared in isopropanol. The absorption was measured continuously at a wavelength of 405 nm in a thermostat-equipped spectrophotometer at 30ºC using sodium phosphate buffer at pH 7.0. The concentration of pNPB in the cuvette was 0.4 mM and the enzyme concentration was chosen so as to allow linear absorbance measurements for 2 minutes. One unit of lipase hydrolytic activity corresponds to the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute. The hydrolytic activity immobilized on Accurel MP1000 was estimated by measuring the lipase activity in the solution before and after adsorption.

Optimal pH, temperature and thermostability of lipase was determined in 0,1 M universal buffer (Britton-Robinson buffer, UB), containing equimolar acetate, ortho-phosphate and ortho-borate [8, 9]. Optimal fatty acid (FA) chain length was determined using various *p*-NP esters at concentrations of 0.3mM in emulsifying buffer, containing 0,1% gum-arabic, 0,2% sodium desoxycholate in pH 8 UB).

Triolein hydrolysis and transesterification with methanol in hexane was carried out in 2ml plastic tubes, final reaction volume was 450µl, which contained 50 µl 0.01 kU lipase solution in buffer and 400 µl 10% triolein (TO) in hexane. Transesterification reaction medium contained TO solution in hexane and methanol of molar ratio 4:1 relative to triacylglycerol. Reaction vials were shaken at a temperature described at 1400 rpm in Biosan Thermoshaker (TS-100). Reaction was stopped by adding equal amount of diethyl ether. Analysis was carried out by thin layer chromatography and densitometry, as described later.

Solvent-free biodiesel synthesis was carried out in 2ml plastic tubes, final reaction volume was 1ml, which contained fat mixture, ethanol (molar ratio 3:1) and 4% (m/v or v/v) lipase. Other conditions were the same as described above.

Ethanolysis The standard reaction conditions were 150 mM triolein and 600 mM ethanol in 2 ml MTBE, in a 4.5 ml glass vial capped with a septum to avoid evaporation. In monoolein alcoholysis, the concentration of monoolein was 50 mM with 200 mM ethanol. The reaction was started by adding 2% (w/v total reaction medium) immobilized lipase preparation. The influence of potential acyl migration catalysts was studied by adding 2% w/v of these to the reaction vial. The reactions were carried out in a thermo mixer at 600 rpm at 30 ºC for 48 hours, unless otherwise stated. Samples were withdrawn by inserting a Hamilton syringe through the septum.

Preparation of monoacylglycerols: 2-monoolein was prepared in a way similar to that described by Millquist et al. [10]. Ethanol (600 mM) and triolein (150 mM) were dissolved in methyl-*tert*-butyl ether (MTBE). Water (0.5%) was added to the reaction mixture. Immobilized RAL (2% (w/v) the amount of enzyme preparation used was based on the total reaction volume) was added to the flask, which was transferred to a reciprocal shaker and shaken for 8 hours at 30ºC, at 160 rpm. Samples were taken for gas

chromatography (GC) analysis to monitor the reaction. The product was further purified as described by Millquist et al. [10] and crystallized from heptane at -20 ºC.

1-monoolein was prepared by esterification of oleic acid with excess glycerol catalyzed by RAL, which is a 1, 3-specific lipase. Substrates were dissolved in *t*-butanol at a concentration of 0.1 M for oleic acid and 0.5 M for glycerol. Immobilized RAL (2% (w/v)) was added to the flask, which was transferred to a reciprocal shaker and shaken for 6-7 hours at 30ºC, at 160 rpm. Samples were taken for GC analysis. The solvent was removed by rotary evaporation under vacuum. The oily residue was dissolved in heptane (30-35 ml heptane/g oleic acid) and centrifuged at 3200 rpm to remove the excess glycerol and water. The supernatant, containing 1-monoolein, was incubated at -20 ºC for 8 hours, and the crystals were separated by vacuum filtration. The purified 1-monoolein (>98% according to GC) was stored at -20ºC.

Phenethyl ester synthesis was carried out in 2ml plastic tubes, final reaction volume was 1 ml, which contained, equimolar initial alcohol and FA concentrations (1M, if not stated otherwise) and 4.2% (m/v or v/v) lipase. Samples were incubated, taken and analyzed as described in trioleino hydrolysis method.

Gas chromatography. The samples containing ethyl oleate, oleic acid, 1-monoolein, 2-monoolein, 1,2-diolein, 1,3-diolein and triolein were analyzed by GC using a Varian gas chromatograph (430-GC-FID, Agilent Technologies Inc., Santa Clara, CALB) equipped with a flame ionization detector and a FactorFour™ capillary column (VF = 1 ms, length 15 m and ID 250 μm, Varian, Agilent Technologies Inc., Santa Clara, CALB). Helium was used as a carrier gas, and the temperature of both the injector and detector was 350 ºC. The starting temperature of the column was 180 ºC. This was maintained for 2.5 min and then increased to 340ºC at 10ºC/min, and then maintained for 26 minutes.

Samples were withdrawn from the reaction mixture and derivatized by silylation by adding an equal volume of *N*-methyl-*N*-trimethylsilyl-heptafluorobutyramide (MSHFBA) and incubating them at room temperature for at least 30 minutes. For the analysis of solvent-free samples and solutions in *t*-butanol, the samples were diluted 10 or 5 times, respectively, before silylation. The silylation reaction was terminated by

adding 99.5% ethanol, at a volume equal to that of MSHFBA. The samples were diluted 15 times with an internal standard solution in cyclohexane to obtain a sample suitable for GC, in which the final concentration of tetradecane was 8 mM. The concentrations of the compounds were calculated using response factors, obtained either from theoretical molar responses [11, 12] or using a standard curve (in the case of triolein).

The initial reaction rates were calculated as the rate of consumption of the substrate per unit weight of protein immobilized on the support (in $\mu mol \cdot min^{-1} mg^{-1}$) during the first hour of the reaction. The concentration of each component was calculated as a percentage of all oleic acid equivalents initially available, taking into account the fact that diacylglycerols and triacylglycerols contain two and three fatty acid moieties, respectively. The yields of ethyl oleate are the maximum yields obtained during the reaction time.

Gas chromatography-mass spectrometry by Perkin-Elmer GC-MS was used to identify the reaction product – phenethyl octanoate (NIST database was used).

Quantitative thin layer chromatography (TLC). Analysis of the reaction products was carried out on TLC plates (5x10cm and 10x10cm) pre-coated with 0.25 mm Silica Gel 60 UV254 (Merck). The sample volumes of 2 µl were applied at a distance of 10 mm from the bottom edge of the plate ant 10 mm between each spot using a 5µl Hamilton syringe. The plate was air-dried and chromatograms were developed in a covered TLC tank using a mobile phase of light petroleum ether (b.p. 40-60 ºC) : diethyl ether : acetic acid (85:15:2), v/v) (a modified method of Šinkūnienė *et al*. [13] and Bendikienė *et al*. [14]. The tank was pre-saturated with mobile phase vapour before use. The developed TLC plates were air–dried for about 10-15 min. 2-PE and PEO spots were visualised under UV light (254 nm), oil spots were visualized after developing the plate in iodine chamber, all spots were visualised after spraying the TLC plates with 0.2% 2,7-dichloroflouresceine solution in 2-propanol and drying on a 30 °C surface for one hour. Pure reference solutions in hexane were used as standards.

Quantitative analysis of reaction products separated by TLC was performed using the principles of densitometry by UVItec Cambridge Fire-reader imaging system and UVItec Fire-reader 15.10 software considering the spot area and colour intensity (spot

volume). The calculations are based on the standard curves of compounds specific absorbance.

Response surface methodology. Experimental design. In order to determine the optimal reaction conditions central composite design (CCD) (TO hydrolysis, transesterification and FEO synthesis) or D-optimal design (transesterification in solvent-free conditions) was employed. The fractional factorial CCD consisted of 4 factorial points, 4 axial points and 5 centre points. The variables selected for the GTO transesterification with 2-phenylethanol were four numeric factors: enzyme concentration (2–7%), substrate concentration (0.8–2M), time (60–120 min); temperature (30–40°C). Variables and their levels selected for the triolein hydrolysis were 2 numeric factors: time (20–180 min); temperature (20–60°C); 1 categorical factor: type of enzyme (Palatase, Lecitase, Lipopan F BG and Lipopan 50 BG).

Data analysis. The experimental data were analyzed using Design Expert 8.01 version and then interpreted. Analysis of variance (ANOVA), a regression analysis and the plotting of response surface were performed to establish optimum conditions for the reaction yield. ANOVA was used to test adequacy and fitness of the responses for linear, 2 function interaction (2fi) and quadratic functions of the variables. A model with P-values (P>F) less than 0.05 was regarded as significant. The lack-of-fit test was used to compare the residual and pure errors at the replicated design points. The highest-order significant polynomial with not significant lack of fit was selected. Predicted residual sum of the squares (PRESS) was used as a measure of fit of the model to the points in the design. After predicting the optimal conditions for synthesis reaction, the experiment was repeated in triplicate to check the reliability of the predicted values and experimental data.

RESULTS AND DISCUSSION

Immobilization and properties of *Enterobacter aerogenes* lipase

The immobilization efficiency dependence on the support nature was studied. Pure and modified chitosan, lignin, polyurethane and Granocell® were the polymers selected

to immobilize lipase by adsorption and covalent attachment. The highest activities were obtained for preparations of covalently immobilized lipase on Granocell 2000® and Granocell 4000® (36 and 38 Units·g^{-1} preparation respectively). Immobilized protein yield was 31-39% in case of PU support, 31% in case of chitosan graft copolymer (S-CHZ) support and 52-53% in case of Granocell® supports (Table 1).

Table 1**.** Covalent (glutar aldehyde activated) immobilization of *Enterobacter aerogenes* lipase on different solid supports: pure polyurethane (PU); macroporous cellulose Granocell - 2000 (Gr-2000) and Granocell - 4000 (Gr-4000); chitosan and poly(ethylene glycol) methyl ether acrylate graft copolymer (CHS-GC)

Carrier	Activity of immobilized preparation, U/g	Activity of immobilized protein, U/mg	Fraction of proteins immobilized, %	Amount of protein immobilized, mg/g
Magnetic lignin	1.9	0.05	44.4	16.5
S-CHZ	7.6	0.92	30.9	8.25
Gr-2000	36.2	6.27	51.8	5.8
Gr-4000	38.8	6.72	52.7	5.8
PU	30.0	2.37	38.6	12.6

A comparative study between soluble and immobilized E. aerogenes lipase is provided in terms of pH and temperature. The enzyme was immobilized onto polymeric carriers by covalent binding. In the case of immobilization of lipase onto PU micro-particles mostly covalent bonds between amino and hydroxyl groups of the enzyme and iso-cyanate groups of PU may cause immobilization. It is usually accepted that reactions between the carbonyl group of bi-functional agent glutar aldehyde and the amino groups of enzymes take place yielding Schiff bases. Due the porous structure of PU as well as graft CHS, adsorption of lipase or entrapment of enzyme into pores of micro-particles could take place, too. The pH and temperature profiles of lipolytic activities are shown in Figures 1 and 2 respectively. The partially purified soluble enzyme was the most active towards p-NPB in mildly alkaline pH range and showed optimum activity around pH 9.0. At pH 7.0 its activity was only about 10% of the maximal activity, at pH 10.0 about 60% of maximal activity was preserved. A slightly lower optimum pH values were found for covalently immobilized on PU (pH 8.5) and magnetic lignin (pH 8.0) E. aerogenes lipase preparations, which also exhibited a relatively higher activity at pH 7.0, showing 70% (lignin-lipase) and 57% (PU- lipase) of the maximal Activity (Fig. 1).

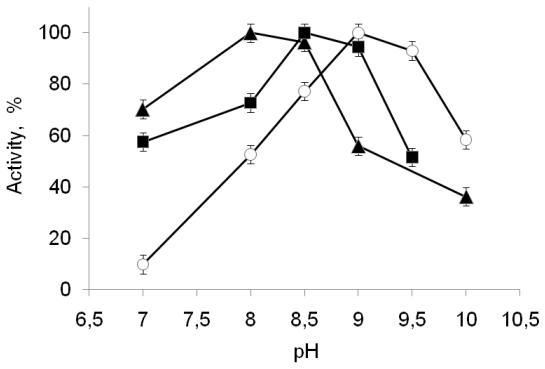

Fig. 1. Influence of reaction pH on *E. aerogenes* lipase activity. Lipase: soluble (○) and covalently immobilized on magnetic lignin (▲) and PU (■).

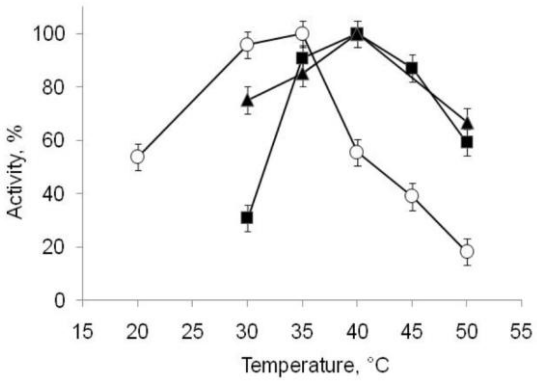

Fig. 2. Influence of reaction temperature on *E. aerogenes* lipase activity. Lipase: soluble (○) and covalently immobilized on magnetic lignin (▲) and PU (■).

The optimal reaction temperature shifted from 35°C for the soluble lipase to 40°C for lipase immobilized on lignin and PU (Fig. 2), implying that the immobilized lipase could be more stable in higher temperatures. However thermal stability was not greatly increased after immobilisation, having 50% of residual activity after 8 min incubation at 60°C (similar to soluble lipase).

Substrate specificity of *Enterobacter aerogenes* 13 lipase. The enzyme specificity was studied with p-nitrophenyl alkanoate esters of varying alkyl chain lengths (C3–C14). The highest hydrolysis rates in case of soluble and immobilized lipase were obtained with p-NP-caprylate (C8), indicating enzymes preference for medium- size acyl chain lengths (Fig. 3). The relative catalytic activities of immobilized enzymes were significantly higher than that of soluble in case of all other esters. Therefore immobilization broadens the enzyme specificity range.

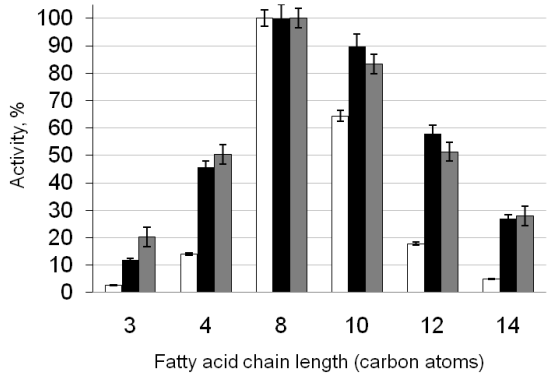

Fig. 3. Relative activity of soluble (■) and covalently immobilized on S-CHZ (■) and PU (□) *E. aerogenes* lipase on different fatty acid *p*-nitrophenyl esters.

Enzymatic synthesis of methyl fatty acid esters is a promising field in biotechnological applications as means for environmental- friendly biodiesel synthesis. The performance of lipase adsorbed on magnetic chitin was studied in the synthesis of methyl-esters. The reaction products were analyzed by TLC after 1, 3, 24, 72 and hours. The greatest yields of methyl ester, monoolein and diolein were reached after 72 hours, whereas the concentration of rapeseed oil in reaction mixture obviously decreased. The existence of methyl ester was detectable after 3 hours of reaction (Fig. 4). After 124 hrs of reaction quantitative evaluation of reaction products has shown 21% oleic acid, 35% methyl oleate, 44% glycerol mono-, di- and tri- esters yield. Therefore a further research and development of *Enterobacter aerogenes* lipase immobilization on natural and cheap supports has perspectives and can lead to a promising Lithuanian-origin lipase catalyst preparation.

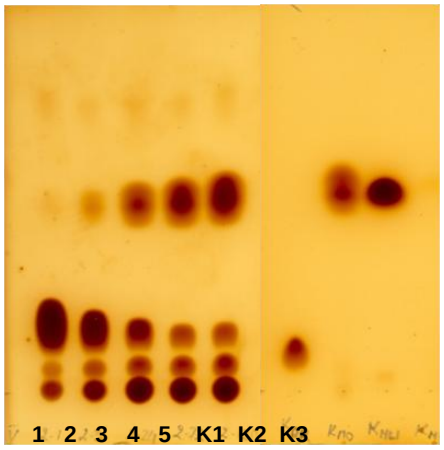

Fig. 4. RAL transesterification with methanol, dependence on reaction time. 10% (m/v) *E. aerogenes lipase* adsorbed on m-CHT (0.3 U), In lanes: reaction mixture after 1–1h; 2–3h; 3–24h, 4–72h; 5–124h. Controls K1 – oleic acid; K2 – methyl oleate; K3 – methyl linoleate.

Triolein hydrolysis and transesterification reaction using response surface methodology

The objective of this section was selection of the most active enzymes for hydrolysis and transesterification reactions and comparison of lipases hydrolytic and synthetic activity in the same reaction conditions (solvent). Literature represents different opinions: some researchers have found that the activity in hydrolysis and transesterification reactions often correlate [15], others imply differently [16], but often lipase activities are compared in different media: aqueous (for hydrolysis) and organic

solvent (for synthesis). Therefore model substrate triolein hydrolysis and synthesis reactions were compared in the same solvent hexane.

The reactions were investigated by means of response surface methodology, thus giving the opportunity to evaluate the effect of several reaction parameters at a time and possibility to compare reaction yields in optimal reaction conditions. The investigated parameters were reaction time (range of 20–180 min) and reaction temperature (range of 30–60°C), using 4 different enzymes (food industry lipases: Palatase®, Lecitase®, Lipopan® F BG and Lipopan® 50 BG).

Triolein hydrolysis

The diagnostics of residuals and influence (Externally Studentized Residuals - Outlier t) helped to detect outliers in the data that were not fit well by the model and which were later disregarded in the analysis (two data points). ANOVA indicated that the second-order polynomial models both for triolein and oleic acid concentrations were statistically significant and adequate to represent the actual relationship between the response and the significant variables, with a model p-value <0.0001.

Final equations for triolein and oleic acid concentrations responses in terms of actual factors are represented in Table 2.

Table 2. Equations in terms of actual factors for triolein and oleic acid responses.

Response	Equation terms					
	Intercept	A (time)	B (temperature)	$AB \cdot 10^{-3}$	$A^2 10^{-3}$	$B^2 10^{-3}$
Y_{1Pal}	110.13	-0.31	-1.40	1.23	1.11	9.43
Y_{1Lec}	97.95	-0.29	-0.74	1.23	1.11	9.43
Y_{1LipF}	113.29	-0.29	-0.81	1.23	1.11	9.43
Y_{1Lip50}	93.08	-0.35	-0.66	1.23	1.11	9.43
Y_{2Pal}	27.43	0.25	-0.55	-0.15	-0.45	6.10
Y_{2Lec}	38.61	0.18	-1.09	-0.15	-0.45	6.10
Y_{2LipF}	10.97	0.11	-0.62	-0.15	-0.45	6.10
Y_{2Lip50}	25.23	0.17	-0.76	-0.15	-0.45	6.10

Y_{1Pal}, Y_{1Lec}, Y_{1LipF}, Y_{1Lip50} correspond to residual triolein concentration after reaction, catalyzed by Palatase, Lecitase, Lipopan F BG, Lipopan 50 BG. Y_{2Pal}, Y_{2Lec}, Y_{2LipF}, Y_{2Lip50} correspond to oleic acid concentration after reaction, catalyzed by Lecitase, Lipopan F BG, Lipopan 50 BG.

Where Y_1 and Y_2 are triolein and oleic acid concentrations respectively, A is time, B temperature and C – enzyme.

The relationships between reaction factors and response can be better understood

by examining the series of contour plots.

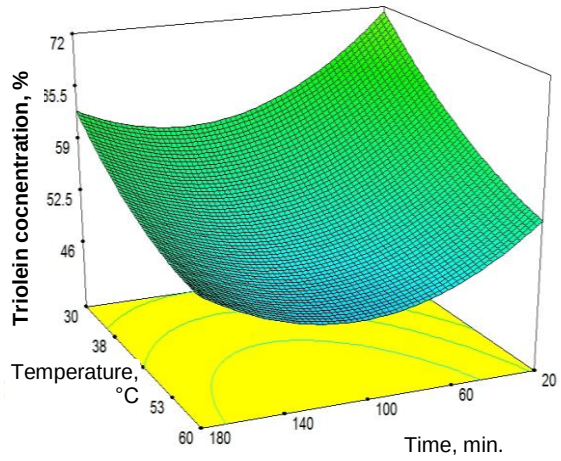

Fig. 5. Response surface plot showing the mutual effect of time and temperature on Palatase - catalyzed triolein hydrolysis (residual concentration, %).

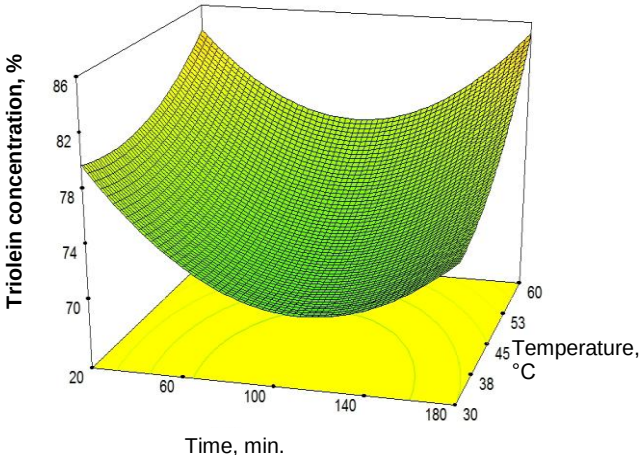

Fig. 6. Response surface plot showing the mutual effect of time and temperature on Lecitase - catalyzed triolein hydrolysis (residual concentration, %)

The optimal reaction time for all enzymes varied between 100 and 140 min, but Lecitase, Lipopan F BG and Lipopan 50 BG preferred lower temperatures (up to 42°C, 48°C and 48°C respectively) (Fig. 6, Fig. 8). Reaction with Palatase exhibited better triolein hydrolysis in higher temperature range of 54-60ºC (Fig. 5). Generally the best hydrolyzing enzyme was Palatase (48% residual trioleino concentration). In cases of other enzymes more residual triolein was left: Lecitase - 72%, Lipopan F BG -84%, 50 BG -60%.

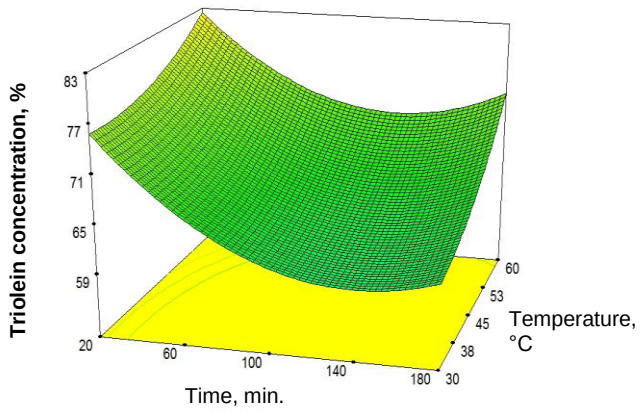

Fig. 7. Response surface plot showing the mutual effect of time and temperature on Lipopan F BG - catalyzed triolein hydrolysis (residual concentration, %).

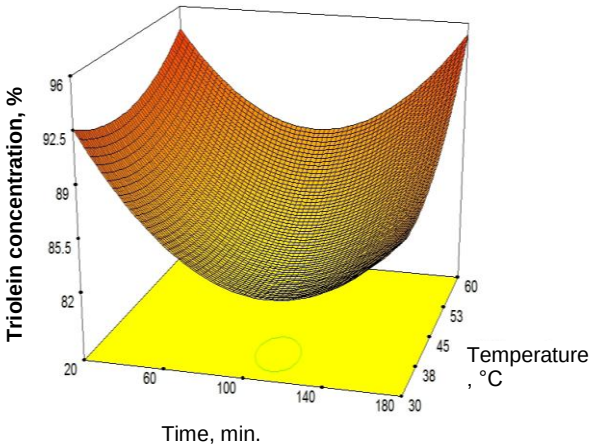

Fig. 8. Response surface plot showing the mutual effect of time and temperature on Lipopan 50 BG - catalyzed triolein hydrolysis (residual concentration, %).

TO concentration is an important parameter concerning an effective fat splitting, disregarding the final reaction products (wastewater treatment, laundry detergents and leather industry). When the aim of hydrolysis is the final reaction product (fatty acid), the degree of triolein hydrolysis is not a sufficient parameter to evaluate the effective reaction, because even when 100% triolein is hydrolyzed, theoretically there might be only 33% of all possible fatty acid moieties formed. Therefore another criterion – oleic acid concentration - was investigated.

A similar pattern is visible in all cases: oleic acid concentration increases with an increase in the reaction time. The highest amount of oleic acid (over 40%) was produced by Palatase (Fig. 9), as shown above it was the best enzyme for triolein hydrolysis as well. In the case of oleic acid formation using Palatase, the effect of varying reaction time is much greater than the effect of varying reaction temperature. This can be due to 1,2- and 1,3-diacylglycerol formation and subsequent hydrolysis. Lecitase and Lipopan 50 BG favoured lower reaction temperatures, but also longer reaction times (Fig. 10 Lecitase, Lipopan follows a similar pattern). In case of Lipopan F BG the concentration of oleic acid was too low to predict effective reaction conditions, though some oleic acid was produced in low temperatures (around 30ºC) at around 120 min.

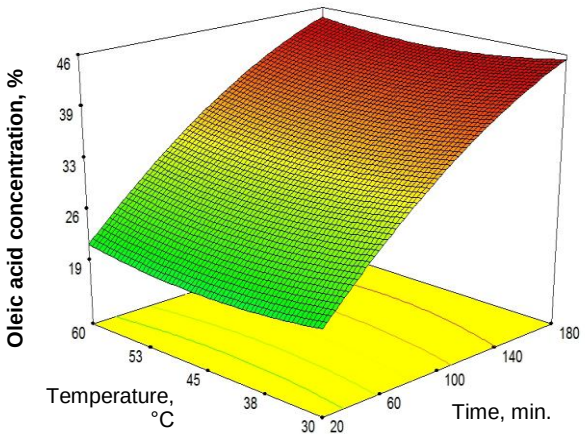

Fig. 9 Response surface plot showing the mutual effect of time and temperature on Palatase – catalyzed oleic acid formation (concentration, %).

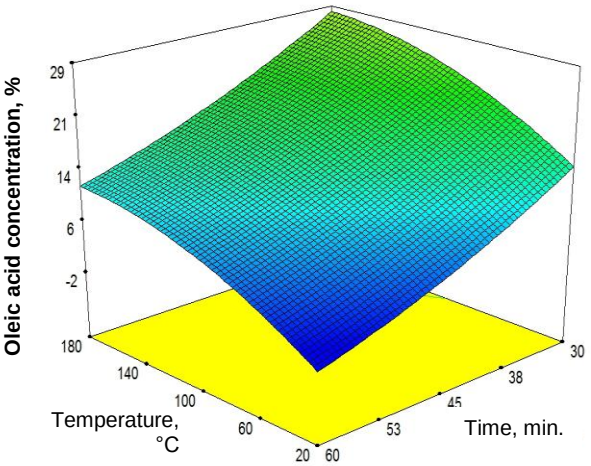

Fig. 10. Response surface plot showing the mutual effect of time and temperature on Lecitase – catalyzed oleic acid formation (concentration, %).

Optimization by Design Expert software was used to determine the optimal conditions for investigated process and was meant to satisfy given criteria: minimize triolein concentration, maximize oleic acid concentration with preference to shorter reaction times and lower temperatures. First two solutions (140 min. reaction time at a

temperature of 50.5 °C, catalyzed by Palatase), were the most appropriate (desirability 0.85). Predicted concentrations were 49.8% TO and 39.8% OR. After repeating the experiment in given conditions the residual TO concentration was 40.4%) and oleic acid concentration was 38.9% which fall into confidence intervals of $CI_{99\%}$ (39.5 – 60.1%) for TO and $CI_{95\%}$ (36.5 – 43%) for OR.

Triolein transesterification with methanol

Experiment design and statistical analysis used were the same as for triolein hydrolysis reaction, described above.

Triolein (Fig. 11) and methyloleate (Fig. 14) responses were described by statistically significant models for Palatase enzyme. Methyloleate formation is temperature-independent, and triolein is split slightly better in higher temperatures (corresponds to triolein hydrolysis data), especially during shorter reaction times.

Table 3. Equations in terms of actual factors for triolein (Y_{1Pal}) and methyl oleate (Y_{2Pal}) responses.

Response	Term				
	Intercept	A (time)	B (temperature)	$AB \cdot 10^{-3}$	$A210^{-3}$
Y1Pal	+116.16	-0.77	-0.50	+3.66	+2.17
Y2Pal	-4.34	+0.35	+0.07	-0.14	-1.17

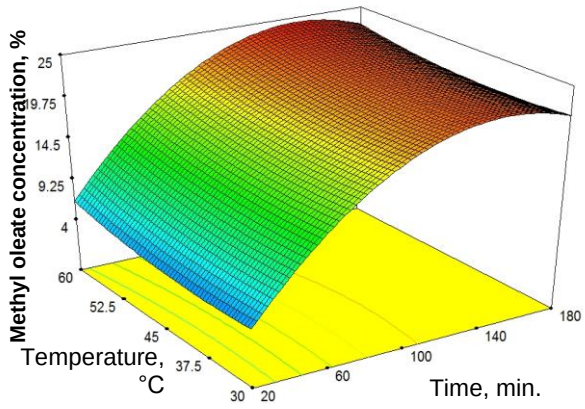

Fig 11. Methyloleate concentration model in TO transesterification in hexane, enzyme Palatase.

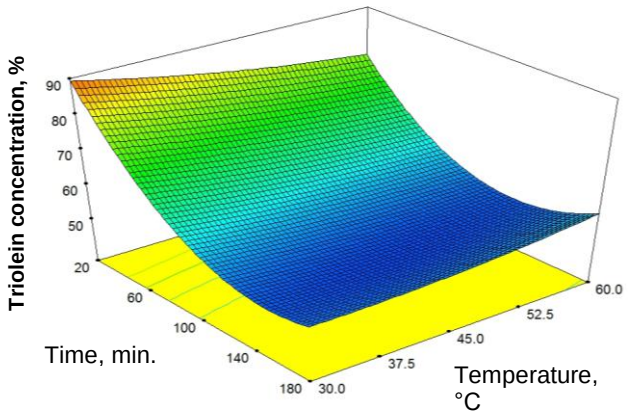

Fig. 12. Triolein concentration model in TO transesterification in hexane, enzyme Palatase.

Lecitase, Lipopan F BG and Lipopan 50 BG enzymes produced too low transesterification reaction yields (MeO yield 1.5%, 2%, 10% respectively), therefore statistically significant RSM models were not possible. Meanwhile Lecitase and Lipopan enzyme preparations were applicable for TO hydrolysis, transesterification reaction could not be catalyzed successfully. Thus correlations between activities and optimal conditions in hydrolysis and transesterification reactions cannot be applied directly.

Optimization by Design Expert software was used to determine the optimal conditions for investigated process and was meant to satisfy given criteria: maximize methyl oleate concentration with preference to shorter reaction times and lower temperatures where the substrate is used to a greater extent. The first solution is 143.5 min. reaction at 30 °C, after repeating the experiment in given conditions, MeO concentration was 26.4%, residual TO concentration was 44.2% TO, that fall into confidence intervals of $PI_{95\%}$, 18.31 - 27.56% for MeO and 40.46 - 62.97% for TO.

Biodiesel synthesis from linseed oil and lard mixture

It is known that linseed oil (LSA) and lard (KT) is an alternative source for biodiesel synthesis instead of pure vegetable oil. LSA is rich in poly-unsaturated fatty acids which in a mixture compensate the properties of saturated fatty acid-rich lard (pork fat). The optimal ratio of LSA and KT in the mixture is 68:32 (m:m) [17]. The enzymes used for screening of transesterification reaction with ethanol (renewable alcohol) were from our laboratory Novozymes© enzyme assortment (Table 4), the reaction was carried out in solvent-free conditions.

Table 4. Enzymes used for enzyme screening for biodiesel synthesis from KT and LSA mixture. Numbers identify enzymes used in lanes in Fig. 13.

	Immobilized/solid		Liquid
1	Lipex 100 T	11	Lipozyme TLL 100 L
2	Novozym 435 FG	12	Lipex 100 L
3	Lipozyme TLL IM	13	Palatase 20000 L
4	Lipopan 50 BG	14	Lipolase 100 L EX
5	Lipopan F BG	15	Resinase A2X
6	Lipolase 100 T	16	Lecitase Ultra
7	Lipozyme RML IM		
8	Novozym 435		
9	Lipoclean 2000 T		
10	Lipoprime 50 T		

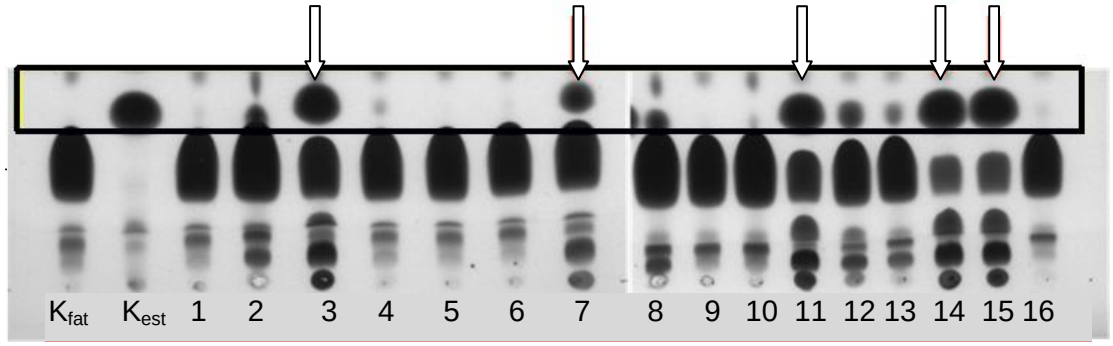

Fig. 13. Thin layer chromatogram of LSA and KT transesterification with ethanol, reaction products after 3 hours. Lane numbering is relative to enzyme numbers in Table 4. K_{fat} – fat, K_{est} - ester controls.

The most effective in screening reactions were *Thermomyces lanuginosus* lipase, both solid and liquid preparations (Lipozyme TLL IM, Lipozyme TLL 100 L, Lipozyme TLL 100 L, Lipozyme 100 L EX, Resinase A2X) and immobilized *Rhizomucor miehei* lipase (Lipozyme RML IM) (Fig. 13). Thus the findings agree with the literature opinion that a number of lipase sources, suitable for biotechnological reactions, is limited [18], moreover, transesterification activity does not correspond to hydrolytic activity, as enzymes actively hydrolyzing p-NPB (Lipopan 50 BG, Lipopan F BG, Lipoclean 2000 T Lipoprime 50 T) were not active in KT and LSA mixture transesterification and *vice versa:* hydrolytically less active enzymes (Lipozyme TLL 100 L, Lipozyme RML IM) were very active in transesterification reactions. RSM (D-optimal design) was employed to carry out a more detailed study for three enzymes: Lipolase 100 L (EX), Resinase A2X and Lipozyme RML IM. Transesterification was studied in the intervals of reaction time 1-8 hours, temperature 30–60 °C, lipase concentration 3–10% (v/v or m/v in reaction mixture) and alcohol:triglyceride molar ratio 4–6.

In the case of Lipolase L (EX) enzyme, reaction temperature had more influence compared to reaction time (Fig. 14), and compared to lipase concentration as well, which is optimal between 6.25 and 8.5% (figure not shown). High alcohol concentration had a positive influence, disregarding the reaction time (Fig. 15) or temperature.

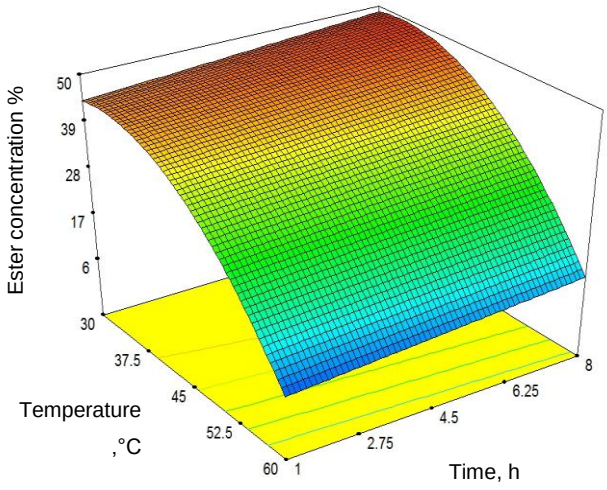

Fig. 14. RSM model for FA ester concentration in LSA and KT mixture transesterification with ethanol, catalyzed by Lipolase L (EX). Influence of reaction time and temperature, alcohol:triglyceride ratio is 5, lipase concentration 6,5%.

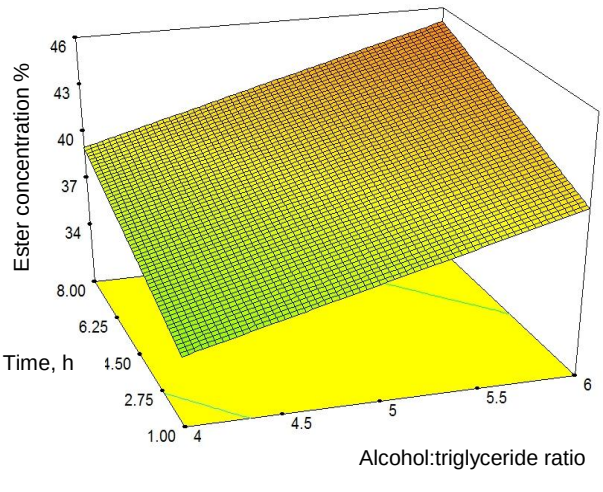

Fig. 15. RSM model for FA ester concentration in LSA and KT mixture transesterification with ethanol, catalyzed by Lipolase L (EX). Influence of reaction time and alcohol:triglyceride ratio, temperature is 45 °C , lipase concentration 6,5%.

Lipozyme RML IM enzyme, similarly to Lipolase L (EX), was more active in lower reaction temperatures (30ºC), but sensitive to increased alcohol concentration, therefore higher yields were expected at lower alcohol concentrations (visual data not shown). Increasing enzyme concentration resulted in increased reaction yield in all range of alcohol concentrations. Soumanou et al. suggested, that in hexane TLL was more active than RML, although in solvent free conditions TLL was almost inactive when methanol was used and RML, being more resistant to alcohol, sustained the activity longer [19]. On the contrary, in this study TLL was not sensitive to ethanol, opposite to RML.

Resinase A2X preparation is very similar to Lipozyme TLL IM, it is also sensitive to reaction temperatures and at alcohol:triglyceride molar ratio equal to 5 and 6,5% lipase concentration the best yields (up to 46%) are achieved in 30–40 °C temperature range, while in 60 ºC the yields of only 10% are expected. Increasing alcohol concentration increases the yield. In any case reaction yield does not vary greatly depending on reaction time; probably accounting to the fast reaction during the first 60 minutes and slower reaction afterwards. The optimal lipase concentration falls into interval of 6.5–9.5%.

Optimization algorithm was applied to maximize the ester yield, by choosing other variables from all range. Optimization solutions for the three enzymes are shown in Table 5.

Table 5. Solutions for optimization of transesterification reaction for LSA and KT mixture with ethanol.

Enzyme	Temperature, °C	Time, h	Lipase conc., %	Alcohol : triglyceride molar ratio	Fatty acid ester concentration
Lipolase L (EX)	33	7.95	6.4	6	52.2
Lipozyme RML IM	30	7.25	10.0	4	42.1
Resinase A2X	35	8.00	8.0	6	49.0

Thus in given conditions using two enzyme preparations (Lipolase L (EX) and Resinase A2X) approximately 50% FA ester concentrations can be achieved. Practically such degree of transesterification is too low, therefore on the basis of optimal reaction conditions an experiment with glycerol removal from reation system and partial alcohol addition was planned. Lipolase L EX enzyme was chosen for its highest predicted yields and insensibility to higher alcohol concentrations. As glycerol removal influences

alcohol and enzyme removal, additional portions were added. In this way 70% ester yield was achieved.

After 20 hours of reaction side-products diglyceride, monoglyceride and free fatty acids are present. The main reason for free fatty acid formation is hydrolysis reaction, which occurs when water is present in a reaction mixture, that is inevitable when liquid enzyme preparations are used. One way to avoid it is usage of immobilized enzyme, which is described in the next chapter (TLL immobilized on polypropylene and commercial Lipozyme TLL IM were used for biodiesel synthesis).

Waste fat, including lard-soybean oil or frying oil blends have been studied as sources for biodiesel fuel by chemical synthesis. The conversions vary between 64 and 89% [20]. Thus previously described enzymatic catalysis is a competitive rival to chemical catalysis, with advantages of mild reaction conditions and possibility to use raw fat resources without additional treatment to remove free fatty acids.

Influence of lipase specificity and acyl-migration on biodiesel synthesis

Since the cost of the raw material constitutes a major part of the production cost of biodiesel, it is very important to achieve a high conversion yield. Enzymatic biodiesel would be more competitive with the chemical alternative if the reactions occurring during enzymatic alcoholysis could be fully understood and optimized. In the alcoholysis of a triacylglycerol substrate, the fatty acids are removed one at a time by the lipase, resulting in alkyl esters and partial acylglycerols and, finally, glycerol, as shown in Fig. 16.

Lipase-catalyzed ethanolysis of a model substrate triolein was studied. Ethanol was chosen instead of the more commonly used methanol, because it can be obtained from renewable resources, and causes less inactivation of the enzymes.

Eight lipases were evaluated as catalysts, immobilized on the same support material, rather than using the commercially available immobilized forms. This allowed a detailed comparison of the enzymes without interferences caused by different support materials.

Most lipases have a preference for the 1- and 3-positions in triacylglycerol substrates, that means that the removal of the fatty acid in the 2-position could constitute a serious bottleneck in the enzymatic biodiesel process. Therefore special attention was paid to the analysis of the isomeric composition of the monoacylglycerols formed during the reaction, and to the effects of potential catalysts of acyl migration.

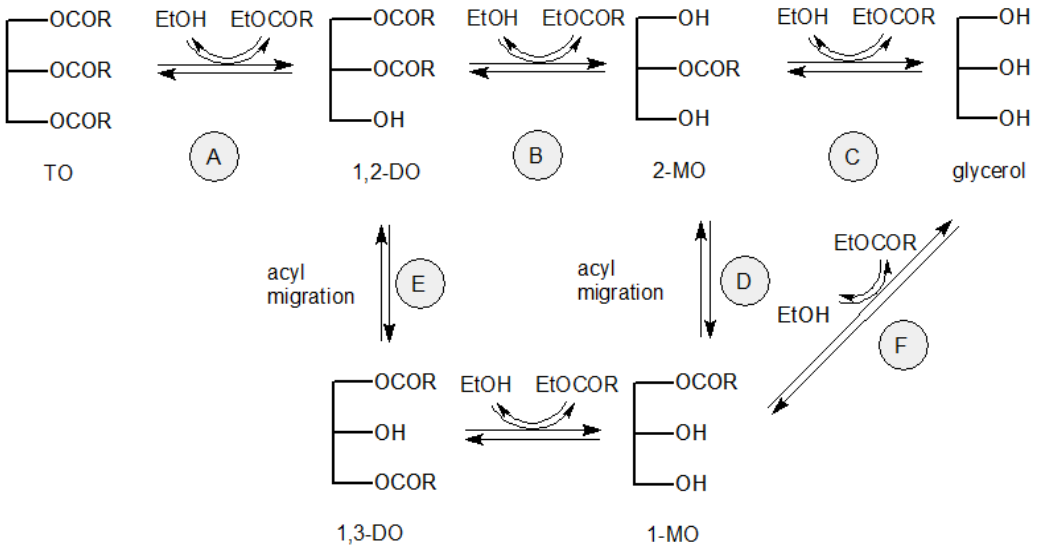

Fig. 16. Reaction scheme for the ethanolysis of triolein. All reactions (A, B, C, and F) except acyl migration (E, D) are lipase catalyzed. Hydrolysis occurs to a small extent (not shown). The upper reaction sequence shows the main reactions in the conversion of triolein to ethyl oleate (EtOCOR). RCOOH = oleic acid. EtOH = ethanol.

The results of lipase immobilization are given in Table 6. In the case of CALB and TLL, most of the protein and the lipase activity were adsorbed on the support. The same protein loading was seen for RALL, about 38 mg/g, which means that only about one third of the protein was adsorbed. The immobilization of lipases on porous polypropylene has been studied previously, and it has been found that Langmuir adsorption isotherms can be used to describe the adsorption process [16]. The amount of lipase on the support leading to saturation depends on the specific surface area available in the pores, and it is probable that 38 mg/g is close to the amount of lipase saturating this support [21, 22]. Almost all lipases were partially purified by immobilization, because the percentage of immobilized activity was substantially higher than percentage of immobilized protein for all lipases except CALB and TLL. In the case of CRL and RMLL, almost all the activity was adsorbed, but only 1.6 and 5% of the protein,

indicating that the lipase commercial preparation contained a large amount of other proteins.

Table 6. Results of lipase immobilization on Accurel MP1000

Enzyme	Immobilized activity (U/g support)	Immobilized protein (mg/g support)	% of activity immobilized	% of protein immobilized
ANL	1.9	0.6	67.1	9.3
CALB	128.7	36.2	94.3	91.3
CRL	375.6	0.089	94.3	1.63
PFL	136.5	15.5	83.4	58.2
RALL	155.4	37.8	63.6	35.4
RNL	2.5	14.2	83	20.9
RMLL	23.3	0.6	90.3	5.1
TLL	982.8	38.5	91.9	80.3

Candida rugosa and *Aspergilus niger* lipases were inactive in triolein ethanolysis reaction and were not studied further.

Influence of reaction additives on triolein ethanolysis reaction

It is known that reaction additives can be beneficial and increase alcoholysis reaction yield [23, 24]. Acyl-migration, independent of lipase catalyst, was studied in 2-monoolein solution in MTBE. Four additives were selected silica gel (it was shown to be beneficial in acyl-migration catalysis [10]), water (in solution with protein and fatty acid it catalyzed acyl-migration [10]), Amberlite IR 120 (H) (sulphonic-groups containing polystyrene polymer) and neutralized form of it (presuming that its chemical properties, *i.e.* sulphonic groups, can catalyze acyl-migration).

The highest acyl-migration rate was obtained with silica gel. (Fig. 17), Amberlite acidic (Amberlite IR 120 (H)) was also effective to some extent, Amberlite neutral (Amberlite IR 120 (Na)) and water (a_w 0.7) did not induce acyl migration. All reaction additives were further investigated in triolein ethanolysis reaction system with enzymes. One of the most important criteria of successful reaction is reaction yield (in this case ethyl oleate concentration). Roughly enzymes could be classified into two categories: the ones for which additives had a positive influence (TLL, RML, and RAL) and the ones for which the influence on ethyl oleate yield was negative (CALB, PFL, and RNL). For instance, using TLL in combination with silica and water resulted in 69.2% and 76.6%

yield after 24 hours, compared to 60.9% without additives. Amberlite acidic had positive influence only in the initial phase of the reaction. The most interesting enzymes TLL, RMLL, RALL and CALB were studied in detail.

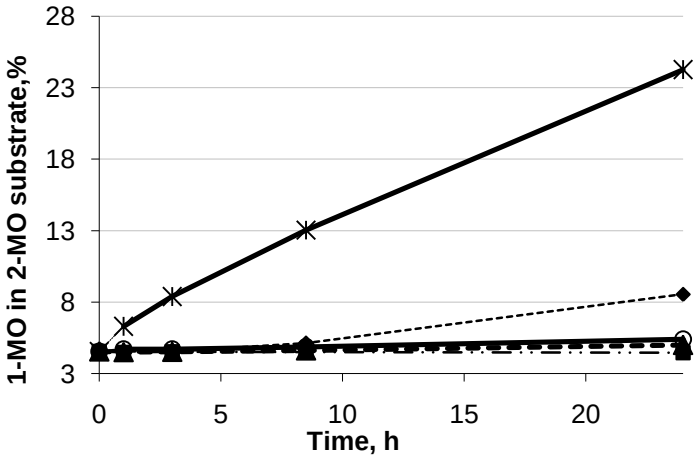

Fig. 17. Acyl migration of 2-monoolein in MTBE with and without additives. The percentage of 1-monoolein in the total monoolein fraction is shown as a function of time. Additives: Ж – silica, ♦ – Amberlite acidic, ▲ – water, ■ – Amberlite neutral, ○ – no additives.

The influence of additives on the initial reaction rate is an important parameter, as for reactions with the same yield the reaction rates can be different while shorter reaction is technologically more beneficial due to the savings of time.

The greatest positive influence of all additives was on TLL-catalyzed reaction initial rate (Fig. 18). Water increases the initial rate almost 4 times (298%), silica gel and Amberlite acidic – around 2.5 times (179 and 153% respectively) and Amberlite neutral by 69%. In the case of RMLL, water increases the initial reaction rate by 59%, other additives have 7–33% positive influence. The additives almost do not affect the reaction rates, catalyzed by RALL (the increase is around 6%). All additives decrease the initial reaction rate, catalyzed by CALB, the highest negative impact is of water (decreased by 98.7%); any form of Amberlite decreases initial reaction rate around twice, and the least negative influence is by silica (decreased by 35%). It is difficult to explain the fact, that all additives had a negative influence on CALB activity, but this phenomenon should be kept in mind while immobilizing CALB on new carriers, which can have a negative influence on enzyme activity. Also using CALB for biodiesel synthesis from low-quality fats and oils can be complicated, because used frying and cooking oils often contain relatively high amounts of water which can inactivate the enzyme.

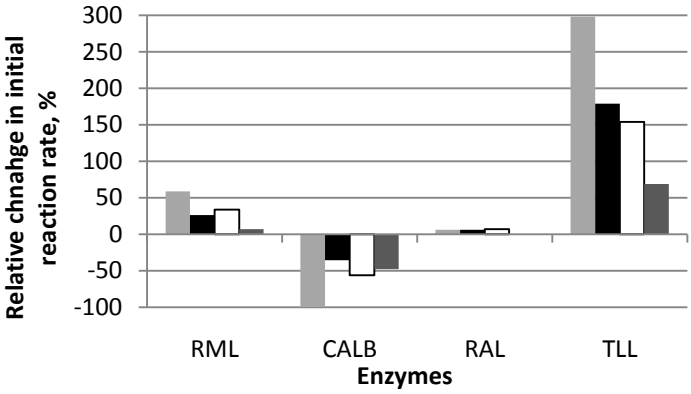

Fig. 18. Influence of reaction additives on the initial reaction rate. The relative change in initial reaction rate in Y axis is based on comparing initial reaction rate with additives to without additives. Additives: ■ - water a_w 0,7, ■ – silica gel, □ – Amberlite acidic, ■ - Amberlite neutral.

The positive influence of water on TLL was described by other researchers [25, 26], though it is rarely considered that higher amount of water is directly influencing the higher yields of by-product oleic acid. In reaction system with water after 48 hours of reaction oleic acid concentration can be higher 10 times compared to the reaction without water (13.2% and 1.32% with TLL respectively). The highest oleic acid concentration is observed when RALL is used (16.29%), the lowest in the case of CALB (2.9%), though it should be kept in mind, that water has a negative influence on CALB activity itself. The addition of silica gel led to an increase in oleic acid formation with all the enzymes studied, and an increase in ethyl oleate yield after 48 hours when using RAL, RML and TLL, but not with CALB, as illustrated in Fig. 19. The highest ethyl oleate yield was obtained using CALB. The main side products were monoolein and diolein in all cases.

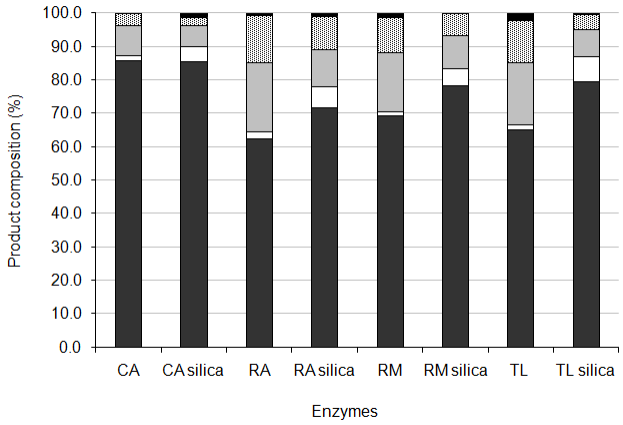

Fig. 19. Product composition after 48 h of ethanolysis of TO catalyzed by lipases with or without silica. From top to bottom: ■ – triolein ▒ – diolein ■ – monoolein □ – oleic acid ■ – ethyl oleate

The alcoholysis reactions revealed that the triolein consumption was rather slow with CALB, and that the amounts of monoolein and diolein were low throughout the duration of the reactions, as can be seen in Fig. 20 A, while the ethyl oleate yield obtained after 48 hours was quite high. These results indicate that CALB converts triolein slowly, but converts monoolein and diolein more rapidly. Among the other lipases, the highest ethyl oleate yield was obtained with TLL when silica gel was added. Rapid consumption of triolein was seen, together with the accumulation of considerable amounts of monoolein and diolein, especially during the early stages of conversion, as shown in Fig. 20 B. Due to the slow conversion of monoolein and diolein, the final ethyl oleate yield was rather low.

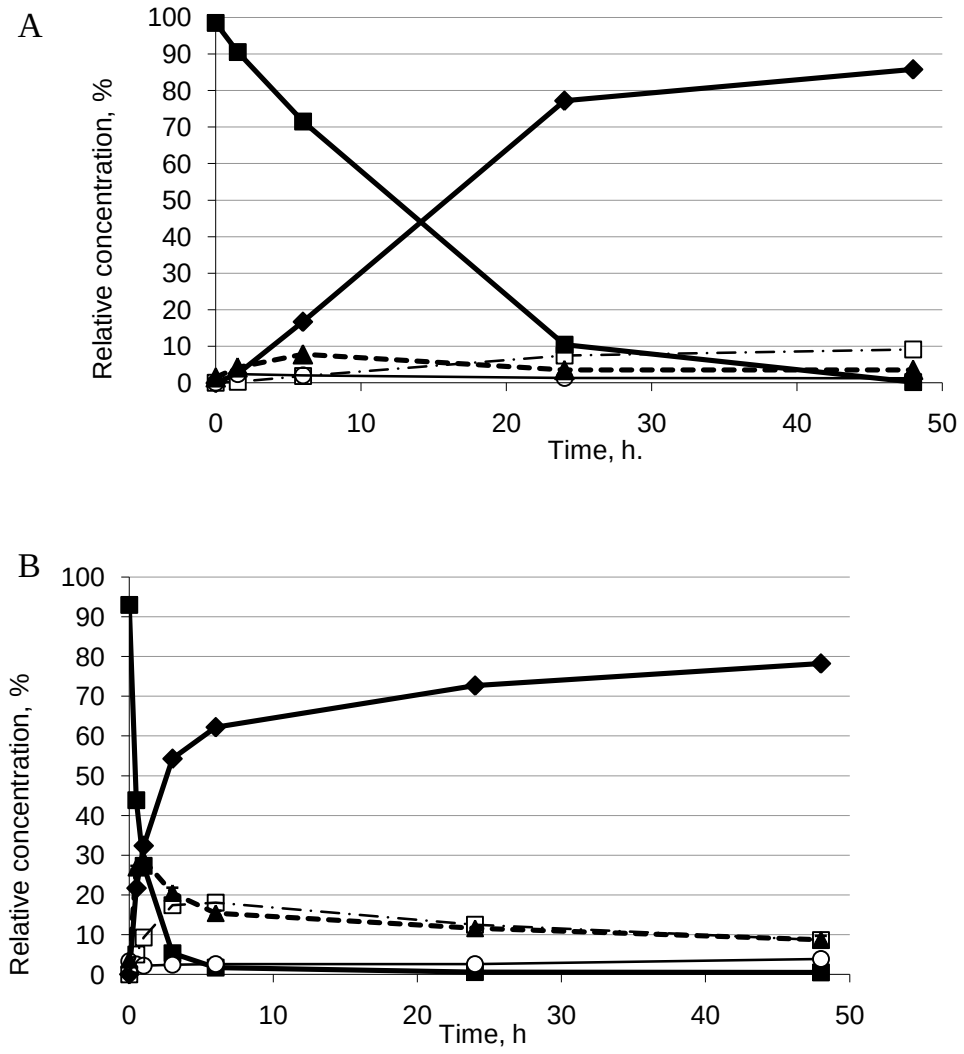

Fig. 20. Time course of TO ethanolysis catalyzed by CALB without additive (A) and TLL with silica additive (B). ♦ – ethyl oleate, □ – monoolein, ▲ – diolein, ○ – oleic acid, ■ – triolein

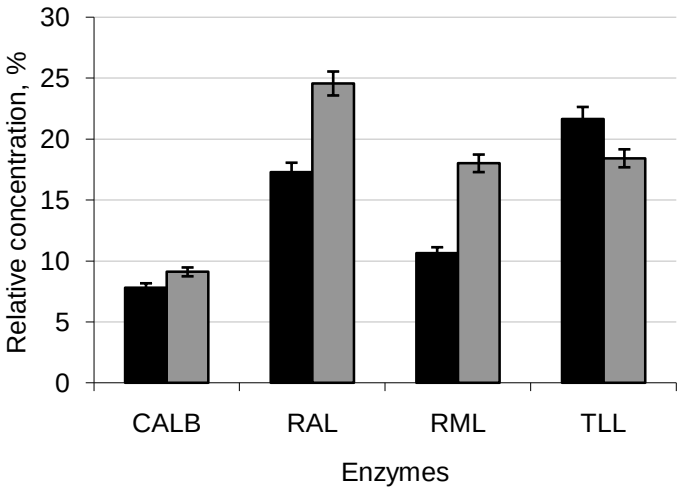

Fig. 21. Maximal amounts of MO and DO in TO ethanolysis. The values shown are expressed as percentages of the oleic acid present in the selected substance among the total amount of oleic acid in all forms. ■ – diolein, ■ – monoolein

Conversions of intermediate reaction products: diolein and monoolein

The concentrations of monoolein and diolein showed maxima in all cases, as can be expected in consecutive reactions. The maximal amounts of monoolein and diolein provide interesting information on the relative rates of conversion of the various acylglycerol substrates by the different lipases. These values were higher for monoolein than for diolein when using CALB, RAL and RML, as can be seen in Fig. 21, which indicates that the third alcoholysis reaction is the slowest in these cases. Only TLL provided a higher maximal concentration of diolein than monoolein. The lowest maximal values for both partial acylglycerols were obtained with CALB, which is clearly very efficient in the alcoholysis of these substrates.

Analysis of the regioisomeric composition of the partial acylglycerols formed in the alcoholysis of triolein revealed that 2-monoolein was the dominating monoolein isomer in the initial phase in all cases. This shows that all the lipases studied have a preference for the 1 and 3 positions in triolein. During the course of the reaction, gradual conversion to the more stable 1-isomer occurred. In most cases, acyl migration was the main mechanism for this isomerisation reaction. In the reaction catalyzed by TLL, the addition of silica gel significantly increased the rate of acyl migration, causing a more rapid decrease in the ratio of 2-monoolein to total monoolein (Fig. 22). However, when using CALB, the addition of silica gel had the opposite effect, slowing down the decrease in the 2-monoolein:total monoolein ratio. In the CALB-catalyzed reaction without silica

gel, a ratio of 2-monoolein:total monoolein of about 11% was reached after 24 hours. This corresponds to the equilibrium mixture between the two regioisomers [27].

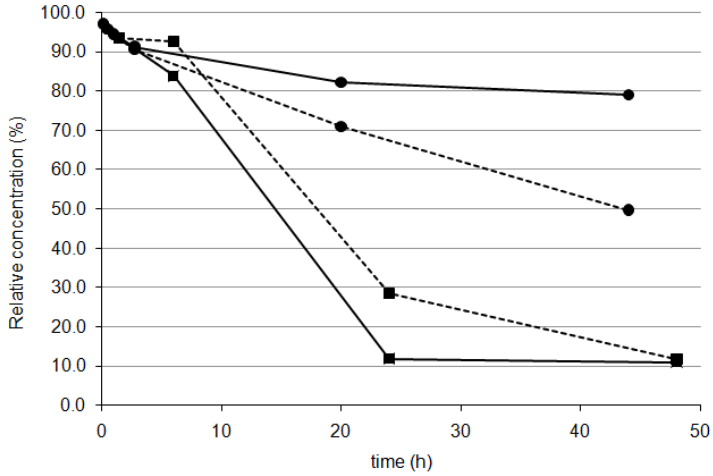

Fig. 22. Ratio of 2-MO to total MO during TO ethanolysis, catalyzed by CALB or TLL, with or without silica. —■— CALB without additives, --■-- CALB with silica,—●— TLL without additives, --●-- TLL with silica

One possible explanation of these observations is that equilibration between the isomers occurred via rapid enzyme-catalyzed conversion of monoolein to glycerol and re-esterification of glycerol to monoolein, according to the scheme shown in Fig. 16. The re-esterification reaction is expected to give mainly 1-monoolein. CALB might be more efficient than the other lipases in catalyzing glycerol esterification. Silica is known to absorb polar substances, including glycerol, and could thus have partly prevented the lipase-catalyzed conversion of glycerol, thereby slowing down the conversion of 2-monoolein to 1-monoolein. Similar effects were observed in the diolein fraction: 1,2(2,3)-diolein was the dominating diolein-isomer formed by all enzymes, and CALB afforded the most rapid isomerisation to the more stable 1,3-isomer, a process that was partly inhibited by silica gel (results not shown). It is thus possible that the CALB-catalyzed alcoholysis and re-esterification pathway also caused isomerisation in the diolein fraction.

In order to obtain more direct information on the conversion of monoacylglycerols, both 2-monoolein and 1-monoolein were synthesized, and alcoholysis of these substrates was studied. Since most lipases have a preference for the 1 and 3 positions in triacylglycerols, the normal triacylglycerol alcoholysis pathway provides 2-monoacylglycerol as a key intermediate, which may accumulate. The relation between

the conversion rates of 2-monoacylglycerol and triolein thus provides information on whether 2-monoacylglycerol conversion constitutes a bottleneck in the overall conversion to ethyl esters. CALB converted 2-monoolein rapidly, while the opposite was observed for the other three lipases studied, as can be seen in Table 7.

Table 7. Reaction rates using different substrates. The substrate concentrations were 150 mM (triolein) or 50 mM (monoolein).

	Initial rates of alcoholysis (μmol·mg-1·min-1)			Rate ratio
Lipase	Triolein	1-monoolein	2-monoolein	2-monoolein/1-monoolein
C. antarctica B	0.29	1.00	1.05	1.05
R. arrhizus	3.75	0.89	0.11	0.12
R. miehei	45.69	57.50	17.64	0.31
T. lanuginosus	1.49	0.58	0.47	0.81

RAL was especially slow in the conversion of 2-monoolein. These results agree with the observation that the maximal accumulated 2-monoolein concentration was much lower when using CALB than the other three lipases (Fig. 21).

The conversion of 1-monoolein was faster than that of 2-monoolein using three of the four lipases, but there was a large variation between the enzymes (Table 7). Only RAL converted 1-monoolein much faster (about 8 times) than 2-monoolein. RML converted 1-monoolein about 3 times faster than 2-monoolein, while the conversion rates were similar for the two isomers when using CALB and TLL. RALL, RMLL and TLL are considered to be 1,3-specific lipases, which agrees with the observations that 1,2(2,3)-diolein was the dominating (>95%) diolein isomer (results not shown), and 2-monoolein the dominating monoolein isomer (results for TLL are shown in Fig. 22) in the initial phase of our alcoholysis reactions. The results for RML and TLL in Table 7 indicate that their regioselectivity is less pronounced for monoacylglycerols than for triacylglycerols, which are the normal substrates on which the classification of 1,3-specificity is based. This also shows that the efficiency of adding acyl migration catalysts to enhance biodiesel synthesis is highly dependent on which lipase is used, and significant improvements can only be expected in some cases.

Step-wise catalysis

Bearing in mind the considerable differences between the specificity of the lipases for different acylglycerols, it has been suggested that combinations of lipases may be beneficial in the biodiesel process. The combination of Lipozyme TLL IM and Novozym 435 has been found to be beneficial in the production of biodiesel from rapeseed oil when *t*-butanol was used as reaction medium [28]. The simultaneous use of about equal amounts of these two enzymes has also been found to be optimal for biodiesel production from lard [29]. A two-step approach, using one lipase in each step, has been described [30], where TLL was used to catalyze the normal alcoholysis reaction, followed by a second step in which CALB, in the form of Novozym 435, was used to catalyze the esterification of free fatty acids in the reaction mixture from the first step. The results obtained in the present study indicate that CALB is the best of the enzymes studied for the conversion of diolein and monoolein, and that the other lipases are more efficient in the conversion of triolein. Therefore, a combination of RAL, RML or TLL during the first phase of conversion, and CALB during the second phase was evaluated. The highest ethyl oleate yield was obtained with the combination of RAL and CALB (

Table 8).

Table 8. Ethyl oleate synthesis using two enzymes consecutively. Step 1: 24 h with RAL, RML or TLL (or TLL with the addition of silica gel), step 2: 48 hours with CALB and two additional portions of ethanol. The reaction temperature was 30 ºC, except in the final case, where it was 40 ºC.

	Amounts of compounds after 72 hours (%) (in oleic acid equivalents)								
Enzymes	Ethyl oleate	Oleic acid	2-mono-olein	1-mono-olein	1,2-di-olein	1,3-di olein	Triolein	Total monoolein	Total diolein
RAL+CALB	94.9	1.2	0.3	3.0	0.2	0.2	0.2	3.3	0.4
RML+CALB	91.6	1.1	0.4	3.0	0.4	1.0	2.5	3.4	1.4
TLL+CALB	91.7	1.1	0.4	2.9	0.4	0.7	2.9	3.3	1.1
TLL+SI+CALB	93.1	2.2	0.4	2.7	0.4	0.3	0.9	3.1	0.7
RAL+CALB 40ºC	95.9	1.1	0.3	2.6	0.1	0.1	0.0	2.9	0.1

RAL is the most 1,3-specific lipase, and is very efficient in converting triolein. It is very important that the first enzyme reduces the triolein concentration to very low levels, as CALB will not decrease it much further. CALB was very efficient in converting diolein, which was reduced to very low levels, while the main side product remaining

was monoolein. The addition of silica gel increased the biodiesel yield slightly when TLL and CALB were used, but RAL and CALB was still the best combination. Increasing the reaction temperature from 30°C to 40 ºC when using the combination of RAL and CALB led to a further increase in yield from 94.9 to 95.9% (Table 8).

Solvent-free reaction

On the basis of results obtained from two-step catalysis in MTBE, reaction was planned in the solvent-free system. Commercial TLL (Lipozyme TL-IM) was used in the first step instead of TLL on polypropylene, because the latter was rapidly inactivated by alcohol (which constitutes a separate phase in solvent-free system) and the second step was carried out with CALB immobilized on Acurell MP 1000 polypropylene. The triolein ethanolysis reaction resulted in 86% ethyl oleate yield after 70 hours. Reactions tend to be slower when solvents are not used and literature examples with high biodiesel yields often describe the usage of 15-25% (sometimes up to 50%) enzyme concentration, column usage, glycerol removal and alcohol addition [26, 31] [32], therefore the degree of conversion in our two-step reaction system during 70 hour reaction time was quite good concerning that enzyme concentration was relatively low (4% m/v in the reaction volume).

The reaction was up-scaled for a natural substrate – rapeseed oil (200 g rapeseed oil used), the reaction products were washed with water to remove glycerol before the analysis. The product contained 84.2% of fatty acid ethyl esters (m/m), over 3% of both monoacylglycerols and diacylglycerols, and around 2% of triacylglycerols (Table 9). Thus the two-step reaction system is a promising approach for biodiesel synthesis, where the yields could further be increased by optimizing the construction of the reactor, enzyme amount/loading, glycerol removal, alcohol addition and other parameters

Table 9. Transesterification of rapeseed oil in solvent-free system, two-step reaction (I step enzyme – Lipozyme TL-IM, II step enzyme - II- CALB).

Reaction products	Concentration (% mass)
FA ethyl esters	84.2
Monoacylglycerols	3.84
Diacylglycerols	3.34
Triacylglycerols	1.98
Glycerol	0

Enzymatic phenethyl octanoate synthesis: optimization by response surface methodology

Flavour esters are widely used in food, fragrance and cosmetic industry, and even though they often occur naturally in plant and animal tissues, the extraction process is too costly and it is rarely applied because of the low ester quantities in the tissues.

Phenethyl octanoate (synonyms 2-phenylethyl octanoate, phenethyl caprylate) is naturally occurring in wines and spirits [33], okra (*Abelmoschus esculentus*) seed coat [34]. It has a mild, fruity, wine-like odour and flavour and can be used in beer, rum, sherry, wine, grape and other fruit flavour applications.

The purpose of this study was to select the appropriate enzyme catalyst and reaction conditions for phenethyl octanoate synthesis from 2-phenyl ethanol (phenethyl alcohol) (PE) and various acyl-donor substrates: octanoic acid (OA) (caprylic acid), glyceryl trioctanoate (GTO) (reaction scheme in Fig. 1) and coconut oil (CCO) (a natural source of octanoic acid).

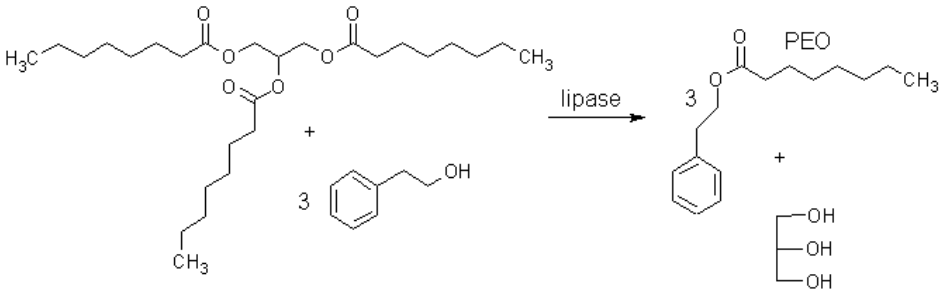

Fig. 23. Reaction scheme of phenethyl octanoate enzymatic synthesis

Enzyme screening and selection

Enzymes selected for screening were commercial lipase preparations Palatase® 20000 L, Lipozyme® RM IM, Lipoclean®, Lipopan® F BG, Lipolase L EX, and non-commercial soluble lipase preparations from *Enterobacter aerogenes* and *Pseudomonas mendocina*.

The screening results are shown in Fig. 2, 2-phenylethyl ester formation is obvious after two hours of reaction time when reaction is catalyzed by Palatase® (lane 3) and

Lipozyme® RM IM (lane 4). A much lower product quantity is observed when Lipolase L EX is used (lane 7).

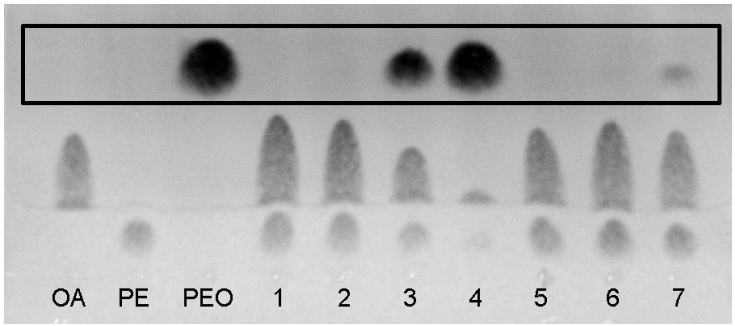

Fig. 24. Lipase screening for 2-phenethyl octanoate (PEO) synthesis reaction: esterification of octanoic acid (OA) with 2-phenylethanol (2-PE). Reaction time: 2 hours, 30°C. Lanes: standards of OA, PE, PEO; reaction catalyzed by Lipopan® F BG (1), Lipoclean® (2), Palatase® (3), Lipozyme® RM IM (4), *Ps. mendocina* lipase (5), *E. aerogenes* lipase (6), Lipolase® 100 L EX (7). Ester products are shown in a box.

There was no esterification activity in cases of Lipopan F BG or lipases from *E. aerogenes and Ps. mendocina*. The presence of 2-PEO was confirmed by means of gas chromatography-mass spectrometry.

After as short as two hours of the reaction high conversion was obtained with *Rhizomucor miehei* lipase in both soluble and immobilized forms, therefore they were selected for further experiments to determine the optimal reaction conditions.

Solvent influence on PEO yield

Two conventional solvents with different polarities were chosen, n-hexane being a non-polar solvent ant *tert*-butanol a polar solvent with 1-octanol/water partition coefficients Log P value of 4.00 and 0.35 respectively. Three different substrates were chosen: OA, GTO and coconut oil. The latter two substrates are representing possible natural substrates for natural (organic) aroma ester synthesis, because triglycerides, containing octanoic acid are found in coconut oil and dairy fat (butter) [35].

Both enzyme preparations have a higher activity and resulting higher conversions in hexane compared to *tert*-butanol, regardless of which substrate is used (Fig. 25).

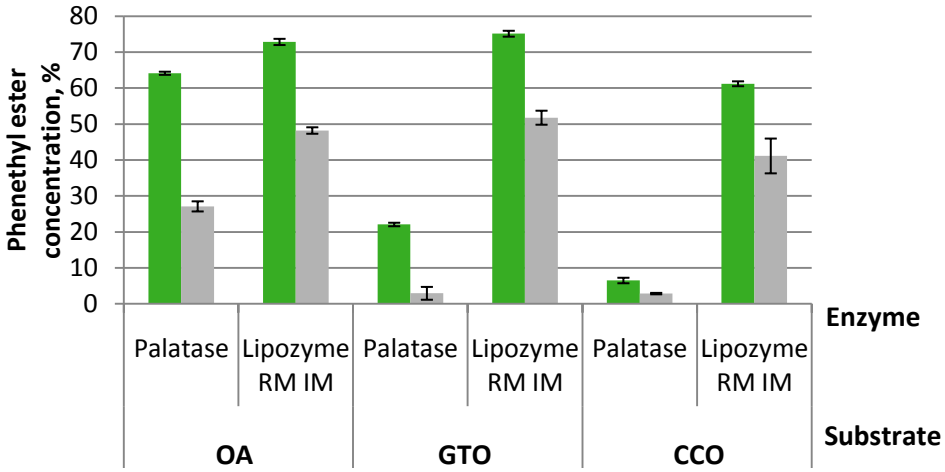

Fig. 25. Solvent influence on octanoate (phenethyl ester) synthesis, catalyzed by soluble (Palatase) and immobilized (Lipozyme RM IM) lipase, using different substrates: OA (octanoic acid), GTO (gliceryltrioctanoate) and CCO (coconut oil). Solvents: ■ – *n*-hexane, ■ – *tert*-butanol. Reaction time - 6 hours.

Although higher ester yields after 6 hours of reaction are obtained in hexane (63%), initial reaction rates depend mainly on the catalyst used, and are higher for immobilized enzyme preparation both in hexane and *tert*-butanol (figures shown in dissertation).

Determination of optimal reaction conditions

Response surface methodology is often used in reaction condition optimization for aromatic ester synthesis. For example, dose aromatic ester (2-phenylethyl acetate) was synthesised enzymatically by transesterification of vinyl acetate with 2-PE and was optimized by RSM, the yields have reached 85,4% in 79 minutes with 4% *Candida antarctica* enzyme (Novozym® 435) for 50mM substrate concentrations in hexane [36].

According the data presented in Fig. 25, it was chosen to optimize phenethyl octanoate synthesis reaction in hexane, catalyzed by immobilized lipase Lipozyme® RM IM, by means of RSM. The influence of four reaction setup factors (enzyme and substrate concentrations, temperature and time) on PEO yield was determined.

The model equation in actual factors is presented in Table 10. The model is highly significant with p-value of <0.0001 and the adjusted multiple correlation coefficient R^2=0.9167. The regression calculations were done by Design Expert program to fit the polynomial models to the selected response. Quadratic model was suggested as the most appropriate and the model was reduced by removing the insignificant terms (with p

values of "Prob>F" greater than 0.1000, indicating model terms are insignificant), the term *BC* was close to the marginal value therefore it was left in the model.

Table 10. Model coefficients for equation in terms of actual factors

Term	Model coefficient
Intercept	+76.85
Enzyme concentration	+10.61
Substrate concentration	-45.14
Temperature	-1.01
Time	+0.12
Enzyme conc.·Substrate conc.	-1.16
Substrate conc.·Temperature	+0.53
Enzyme conc.2	-0.57
Substrate conc.2	+6.24

In Fig. 26 the influence of enzyme and substrate concentrations on the reaction yield is plotted: the higher the enzyme concentration and the lower the substrate concentration, the higher is reaction yield (Fig. 26), as it is also seen in Table 10, that the influence of enzyme concentration is positive and the influence of substrate concentration is negative, which could be due to the enzyme saturation by substrate.

In the temperature of 35 °C and 90 minutes of reaction time the values vary from 37% (enzyme concentration 2%, substrate concentration 2M) to 75% (enzyme concentration 7%, substrate concentration 0.8 M).

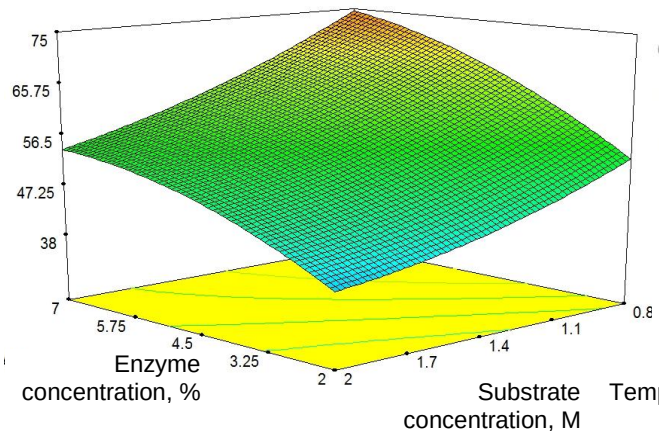

Fig. 26. Response surface plot showing the mutual effect of substrate and enzyme concentrations on the reaction yield (conversion, %), temperature and time are set to mean values (i.e. 35 °C and 90 min).

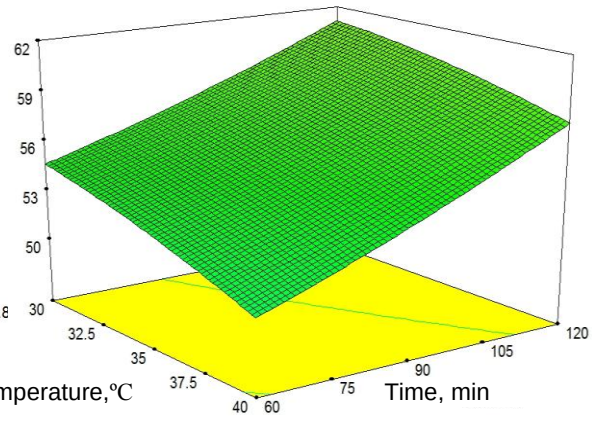

Fig. 27. Response surface plot showing the mutual effect of reaction temperature and time on the reaction yield (conversion, %), enzyme and substrate concentrations are set to mean values (i.e. 4.52% and 1.4 M respectively).

The effect of reaction temperature is also negative, that means that the better catalysis is achieved in lower temperatures. The effect of reaction time is positive, and, even when approaching 120 min the yield is still increasing (Fig. 27). Therefore it should be considered that longer reaction times (if they are acceptable) might increase the yield.

Optimal conditions

The optimal conditions of Lipozyme® RM IM-catalyzed PEO synthesis were predicted using the Design Expert numerical optimization tool. The target was to maximize PEO yield by choosing the optimal variable values from all response surface design range. The optimal conditions for GTO transesterification with PE were: enzyme concentration = 7%, substrate concentration = 0.8 M, Temperature = 30 °C, Time = 120 min, the predicted yield was 81.1%. To validate the model the experiment was repeated at given conditions and the yield was 80.0%, the results correlate well with the predicted data.

Kim *et al*. have synthesized various PE flavour esters enzymatically, using enzyme mixtures. PEO yield was 64% after 20 hours using 10 mM OA, 20 mM PE and 2mg/ml of each enzyme (*Penicillium roqueforti*, *Candida rugosa* and *Aspergillus niger*) in hexane [37].

Our optimized reaction offers higher conversion and also higher productivity, because higher substrate concentrations are used. 80% conversion can be obtained in as short as two hours, but the concentration of enzyme catalyst is higher (7%) than in the previous study.

Other substrates. It was confirmed that RML can be used in esterification of long chain fatty acids (C18, C18:1, C22, C22:1) in *tert*-butanol and transesterification of coconut oil with 2-phenyl ethanol in solvent-free conditions, therefore it can be concluded, that Lipozyme RM IM lipase preparation is a versatile enzyme for esterification and transesterification reactions with aromatic alcohol 2-phenylethanol.

Conclusions

1. Immobilization of *Enterobacter aerogenes* lipase on natural and/or cheap carriers improves enzyme's catalytic properties.
2. The best out of studied enzymes to catalyze triolein hydrolysis in hexane is commercial preparation Palatase®. Most promising enzymes for fatty acid ester synthesis are lipases from *Rhizomucor miehei, Thermomyces lanuginosus, Rhizopus arrhizus, and Candida antarctica* (B) microorganisms.
3. Silica gel is an effective additive for transesterification catalysis when *Thermomyces lanuginosus*, *Rhizomucor miehei* and *Rhizopus arrhizus* lipases are used, though suppresses the reaction catalyzed by *Candida antarctica* lipase B.
4. The investigated lipases have pronounced differences in their specificity to different acylglycerol classes and different acylglycerol regioisomers. The 1,3-specificity for monoacylglycerols is more pronounced than for triacylglycerols.
5. It is purposeful to catalyze biodiesel synthesis stepwise: first step is catalyzed by enzyme, fast in triacylglycerol splitting, second is catalyzed by enzyme, fast in transesterification of di- and monoacylglycerols. Optimal combination is *Rhizopus arrhizus* and *Candida antarctica* B lipases.
6. The most appropriate catalyst for phenethyl octanoate synthesis is a commercial lipase Lipozyme RM IM® in hexane. Higher conversion is achieved in lower temperature range and lower substrate concentrations.

List of publications

1. Šinkūnienė D, Bendikienė V, Juodka B (2011) Response surface methodology-based optimization of lipase-catalyzed triolein hydrolysis in hexane. Rom Biotech Lett 16:1–11.
2. Šinkūnienė D, Kiriliauskaitė V, Bendikienė V, Juodka B (2008) Properties and Immobilization of Enterobacter aerogenes 13 Lipase. Rom Biotech Lett 13:1–8.

Publications in non-peer-reviewed journals:

1. Kiriliauskaitė ,V., Šinkūnienė ,D., Bendikienė V. Lipazių katalizuojamų biotechnologijai svarbių procesų tyrimas. Konferencijos "Mokslas Gamtos mokslų fakultete" pranešimai. Vilnius. 2010 m. spalio 22. P. 74-84

Conference presentations

1. D. Šinkūnienė, V, Bendikienė, P. Adlercreutz. The influence of reaction additives on biodiesel synthesis catalyzed by immobilized *Thermomyces lanuginosus* lipase. Conference „Doctoral internships in science centers abroad". October 11. Abstract in conference proceedings PP. 62-65.
2. V. Kiriliauskaitė, D. Šinkūnienė, V. Bendikienė. Lipazių katalizuojamų biotechnologijai svarbių procesų tyrimas. „Science in the Faculty of Natural Sciences, Vilnius university". October 22, 2010, Vilnius.
3. XI International Congress of Lithuanian Biochemical Society, Tolieja "LBS 50". D. Šinkūnienė, V. Bendikienė, B. Juodka. Optimization of enzymatic triolein hydrolysis using responce surface methodology. June 15-17 d.2010, Tolieja (Molėtų raj.). Abstract in conference proceedings P. 55.
4. D. Šinkūnienė, V. Kiriliauskaitė, V. Bendikienė, B. Juodka. Properties and Immobilization of *Enterobacter aerogenes*-13 Lipase. International conference on industrial microbiology and applied biotechnology. October 9-11, 2008, Galati, Romania. Abstract in conference proceedings P. 19.

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Reziume

Lipazių katalizuojama sintezė yra perspektyvi alternatyva cheminei sintezei biodyzelino, kvapiųjų junginių bei kitų sudėtingos sandaros junginių atveju. Šiame darbe buvo siekta ištirti ir optimizuoti riebalų rūgščių sintezės, panaudojant lipazes, būdus.

Vienas iš fermentų efektyvaus panaudojimo būdų yra jų imobilizavimas. Lietuvoje išskirto *Enterobacter aerogenes* – 13 kamieno lipazė sėkmingai imobilizuota ant įvairių Lietuvoje susintetintų gamtinių ir polimerinių nešiklių. *Enterobacter aerogenes* – 13 preparatai, imobilizuoti kovalentiniu būdu, naudojant glutaro aldehidą, pasižymėjo didesniu aktyvumu, nei adsorbuoti. Ant poliuretano ir makroporingos celiuliozės (Granocell) nešiklių imobilizuoti preparatai buvo aktyviausi (30-38 U/g), be to, imobilizavimas pagerino preparatų savybes: švelniai šarminėje aplinkoje aktyvios tirpios lipazės pH optimumas po imobilizavimo ant poliuretano bei ant įmagnetinto lignino pasislinko link neutralių pH verčių, optimali temperatūra padidėjo nuo 30 ºC iki 40 ºC, o lipolizinis aktyvumas riebalų rūgšties grandinės ilgio atžvilgiu išsiplėtė, tačiau visais atvejais greičiausiai buvo hidrolizuojamas C8 RR grandinę turintis *p*-nitrofenilo esteris. Ant patogaus naudoti magnetinio chitino imobilizuotas *Enterobacter aerogenes* lipazės preparatas buvo tinkamas rapsų aliejaus peresterinimo metanoliu reakcijai katalizuoti. Tirta lipazė ir nešikliai bei imobilizavimo metodai yra perspektyvūs, todėl tęsiant tyrimus būtų galima tikėtis sukurti dideliu aktyvumu pasižymintį lietuvišką imobilizuotos lipazės preparatą.

Komercinių preparatų atrankos bei optimizavimo metu, tiek hidrolizės, tiek sintezės reakcijoms, išskirtinėmis savybėmis pasižymėjo šie fermentai: Palatase (*Rhizomucor miehei* lipazė) – buvo efektyviausias TO hidrolizės ir transesterinimo metanoliu heksane reakcijos katalizatorius, Lipozyme TL IM, Lipolase L (EX) (*Thermomyces lanuginosus* lipazė) Lipozyme RM IM (*Rhizomucor miehei* lipazė) ir Resinase A2X (*Thermomyces lanuginosus* lipazė) – linų sėmenų aliejaus ir kiaulių taukų mišinio peresterinimui be tirpiklio, Lipozyme RM IM – fenetiloktanoato sintezei heksane. Be TLL ir RML, *Rhizopus arrhizus* lipazė ir *Candida antarctica* lipazė B pasižymėjo efektyviu trioleino tranesterinimui etanoliu metil-*tret*-butil-eteryje. Todėl pradedant tirti naujas fermentinės esterių sintezės reakcijas reikėtų nepamiršti į fermentų atrankos sąrašą įtraukti minėtų

lipazių, pasižyminčių dideliu aktyvumu ir skirtingu specifiškumu bei atsižvelgti į darbe aprašytas jų savybes bei optimalias reakcijų sąlygas.

Reakcijos priedų įtaka trioleino etanolizės (biodyzelino sintezės) reakcijoms yra nevienareikšmiška, priklausomai nuo to, kuris fermentas naudojamas katalizei. Silikagelis padidino reakcijos išeigą bei pagreitino acilglicerolių regioizomerų izomerizaciją naudojant RML, TLL ir RAL, tačiau sumažino, naudojant CALB. Galbūt tai vyko dėl glicerolio adsorbcijos ant silikagelio ir izomerizacijos, vykstančios grįžtamai esterinant glicerolį, slopinimo. Vanduo padidino etiloleato išeigą bei pagreitino acilglicerolių izomerizaciją RML ir TLL, atveju, tačiau visų fermentų atveju lėmė žymiai didesnę šalutinio reakcijos produkto – oleino rūgšties – koncentraciją. Amberlite IR 120 H reakcijos priedas slopino acilo grupės migraciją, jis galėtų būti naudingas regioselektyvios sintezės reakcijoms.

Lipazės ryškiai skyrėsi savo specifiškumu skirtingoms acilglicerolių klasėms ir skirtingiems acilglicerolių regioizomerams. Tai turėjo esminės įtakos biodyzelino susidarymui. Įdomu tai, kad fermentų regioselektyvumas buvo mažiau išreikštas monooleino, nei trioleino atžvilgiu.

Dėl lipazių specifiškumo skirtumų pasirodė naudinga jas naudoti kombinacijomis: pirmą reakcijos etapą, kuriame pagrindinis reakcijos substratas yra triacilglicerolis, katalizuojant fermentu, greičiau skaldančiu triacilglicerolių esterinius ryšius (RAL, TLL, RML), o antrą etapą, kuriame pagrindiniai substratai yra diacilgliceroliai ir monoacilgliceroliai, katalizuojant greičiausiai šiuos junginius peresterinančiu fermentu (CALB). Vykdant dviejų etapų reakciją ir naudojant po 2 % RAL ir CALB preparatų, pasiekta 96 % esterių koncentracija.

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