VILNIUS UNIVERSITY

VITA KIRILIAUSKAITĖ

EXTENDED INVESTIGATION OF LIPASES FOR BIOTECHNOLOGICAL APPLICATION

Summary of doctoral dissertation

Physical sciences, Biochemistry (04 P)

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Scientific supervisors:
Prof. habil. dr. Benediktas Juodka (Vilnius University, Physical Sciences, Biochemistry – 04P). 2010 10 01 - 2012 12 27;
Dr. Vida Bendikienė (Vilnius University, Physical Sciences, Biochemistry – 04P).
2012 12 28 - 2014 09 30.

Evaluation board of dissertation of Biochemistry field:

Chairman Prof. Dr. Kęstutis Sužiedėlis (National Cancer Institute, Physical Sciences, Biochemistry – 04P).

Members:
Dr. Sigita Jurkonienė (Nature Research Centre, Biomedical Sciences, Biology – 01B);
Prof. Habil. Dr. Albertas Malinauskas (Center for Physical Sciences and Technology, Physical sciences, Chemistry – 03P);
Prof. Dr. Tautgirdas Ruzgas (Malmö University, Sweden, Physical sciences, Biochemistry – 04P);
Assoc. Prof. Dr. Saulius Serva (Vilnius University, Physical sciences, Biochemistry – 04P).

The official defence of the dissertation will be held at the Great Auditorium of the Faculty of Natural Sciences, Vilnius University, at 14 p.m. on 17 of March, 2015.

Address: M.K. Čiurlionio 21/27, LT-03101, Vilnius, Lithuania

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VILNIAUS UNIVERSITETAS

VITA KIRILIAUSKAITĖ

BIOTECHNOLOGINĖS PASKIRTIES LIPAZIŲ TAIKYMO IŠPLĖSTINIS TYRIMAS

Daktaro disertacijos santrauka

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Moksliniai vadovai:

Prof. habil. dr. Benediktas Juodka (Vilniaus universitetas, fiziniai mokslai, biochemija – 04 P). Nuo 2010 10 01 iki 2012 12 27;
Dr. Vida Bendikienė (Vilniaus universitetas, fiziniai mokslai, biochemija – 04 P).
Nuo 2012 12 28 iki 2014 09 30.

Disertacija ginama Vilniaus universiteto Biochemijos mokslo krypties taryboje:

Pirmininkas – prof. dr. Kęstutis Sužiedėlis (Nacionalinis vėžio institutas, fiziniai mokslai, biochemija – 04P).

Nariai:
dr. Sigita Jurkonienė (Gamtos tyrimų centras, biomedicinos mokslai, biologija – 01B);
prof. habil. dr. Albertas Malinauskas (Fizinių ir technologijos mokslų centras, fiziniai mokslai, chemija – 03P);
prof. dr. Tautgirdas Ruzgas (Malmö universitetas, Švedija, fiziniai mokslai, biochemija – 04P);
doc. dr. Saulius Serva (Vilniaus universitetas, fiziniai mokslai, biochemija – 04P).

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INTRODUCTION

The manufacturing process of various daily use products, such as paper, textiles, food, feed, fuel, chemicals and pharmaceuticals, not only consumes a large amount of raw materials and energy, but also generates a lot of wastes of different origins having a negative impact on the environment and the quality of life. The growth of a global human population and economic development are the main reasons that determine increased consumption and environmental damage caused by traditional chemical technologies. Therefore, various alternative technologies are being developed that will allow to meet the growing demand of various products, consuming lower levels of raw materials and minimizing the environmental pollution problems.

Industrial biotechnology is currently one of the fastest-growing industrial sectors not only in Europe but all over the world, using different biotechnological methods for the production of a variety of materials. The main objective is the gradual replacement of oil with renewable raw materials, since the development of industrial biotechnology is promoted not only by various environmental problems (the increasing environmental pollution, the greenhouse effect) but also by limitations of petroleum reserves.

One of the main tools for biotechnological production are enzymes - biological catalysts - with limitless application capabilities. Due to their versatility lipases (triacylglycerol acylhydrolases, EC 3.1.1.3.) are among the most investigated enzymes widely used for the synthesis of various compounds. The physiological function of lipases is associated with the lipid metabolism, however, depending on the reaction conditions, these enzymes can catalyze a variety of biotechnologically important reactions, including ester synthesis, aminolysis, epoxidation, lactonization, polymerization, etc.

It has been known for a long time that, in comparison with the usual traditional chemical catalysis, enzymatic synthesis has some great advantages, including mild reaction conditions, a high substrate specificity, absence of adverse reactions, the products of higher quality, eco-friendly and non-toxic processes. The application of such bioprocesses, producing a variety of bio-based products suitable for everyday use, could ensure sustainable economic development.

On the other hand, in order to obtain optimum yields of the desired products, a large quantities of enzymes are used. Moreover, after a several reaction cycles the reduction of enzymatic activity is usually determined. In addition, enzymes can be inhibited by the components of the reaction mixture, enzymatic processes take a relatively long time, and for these reasons enzymatic synthesis is not as cost-effective as it is expected. Therefore, in order to reduce the cost of enzymatic production, it is necessary not only to determine the optimal reaction conditions, but also to select an appropriate enzyme with suitable activity and specificity upon different substrates during different reactions under various conditions. Therefore, during the dissertation work the activity and specificity of several commercial lipases was investigated. Lipase-catalized reactions were carried out under different conditions to form various biotechnologically important products, necessary for our daily life – biodiesel, biolubricants, multi-purpose esters.

The main objective of this research was to investigate the possibilities of commercial lipases application for the synthesis of biotechnologically important esters.

The research was aimed at:

1. Investigation of the effects of various reaction conditions on the productivity of biodiesel synthesis catalyzed by commercial lipases.
2. Evaluation of the ability of various commercial enzymes to catalyze the synthesis of complex esters – biolubricants - when one of the substrates is trimethylolpropane (TMP).
3. Selection of new substrates for lipase-catalyzed synthesis of various valuabe esters and evaluation of the influence of various parameters on process productivity.
4. Investigation of soap manufacturing wastes – suds – utilization capabilities via transesterification/esterification processes catalyzed by lipases.

Scientific novelty

Although the enzymatic production of biodiesel is not a new area of research, optimal conditions for different enzymes can vary drastically, for this reason each new enzyme must be investigated separately. During this work commercial lipase Lipoprime 50T-catalyzed rapeseed oil (RO) transesterification with methanol (methanolysis) was investigated in detail for the first time. In addition, it was also evaluated the influence of ectoine (new additive for biodiesel production) and new promising solvents (glycol ethers, glymes) on the catalytic activity of various lipases preparations.

The synthesis of biolubricants when one of the substrates is TMP, is usually carried out under reduced pressure, using extremely high amounts of the enzyme, while in this work the research performed in order to optimize the process for the industrial production without the need of sophisticated equipment and with reduced amounts of the enzyme required.

The selection of different oils during transesterification with terpenols (β-citronellol and geraniol) for the synthesis of valuable esters used in biotechnology was carried out for the first time. Moreover, during this investigation glutaric acid and β-citronellol diester was obtained. The details about enzymatic production of this compound has not been described in literature yet and all complex chemical methods are patented. Moreover, it was determined that during lipase-catalyzed oxalic acid esterification reaction with oleyl alcohol and β-citronellol different number of compounds is produced, which can vary among different commercial lipolytic enzymes as well.

During the work new fatty waste utilization reseach was carried out. The soap manufacturing wastes – suds – were enzymatically transesterified/esterified with various alcohols forming different esters.

Doctoral dissertation contents

Original doctoral dissertation (in Lithuanian) contains the following parts: Introduction, Literature review, Materials and Methods, Results and Discussion, Conclusions, Reference list (452 references cited), List of publications (3 ISI WOS papers), Participation in conferences (6 events), Acknowledgements, 73 Figures, 11 Tables, 158 pages in total.

MATERIALS AND METHODS

Materials. Commercial „NovoNordisk“ (Lipoprime 50T) and „Novozymes“ (all except of Lipoprime 50T) preparations of lipases were kindly donated by Novozymes representatives in Lithuania – JSC „Biopolis“ (Vilnius). Most oils used for investigations were purchased in local food markets, except of castor oil (CsO), purchased in a pharmacy, coconut oil (CcO), obtained from Sigma, and false flax oil (FFO) which was extracted from plants grown in local experimental fields (Aleksandras Stulginskis University, Kaunas, Lithuania). All other chemicals used in the study were products of analytical grade.

Determination of protein content was performed by Bradford assay [1] using bovine serum albumin for a standard calibration curve.

The lipolytic activity of liquid lipase preparations was determined using a spectrophotometric assay based on the hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB), measuring the change of optical density at 410 nm. The reaction was conducted in thermostated cuvette at a constant temperature of 30^{o}C. The reaction mixture consisted of pH 8.0 100 mM universal buffer (UB, Britton – Robinson buffer; composed of acetic, ortho-boric and ortho-phosphoric acids at a ratio of 1:1:1), 10 mM *p*-NPB solution in 2-propanol and a certain amount of enzyme. The final concentration of *p*-NPB in the cuvette was 0.1 mM. Blank sample without enzyme was measured to evaluate self-hydrolysis of the substrate [2]. One unit of lipase hydrolytic activity (U) corresponds to the amount of the enzyme releasing 1 μmol of *p*-nitrophenol per minute.

The hydrolytic activity of immobilized lipase preparations was determined using the same method as described above based on the hydrolysis of *p*-NPB, measuring the change of optical density at 410 nm after 1 – 3 min (depending on the activity of lipase). The reaction mixture consisted of pH 8.0 100 mM 2.5 – 7.5 ml UB, 25 - 75 μl 10 mM *p*-NPB solution in 2-propanol and 20 mg of immobilized enzyme preparation. The enzyme was separated from the reaction solution by filtration before each measurement.

The hydrolytic activity of liquid lipase preparations was also determined using a spectrophotometric assay based on the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP), measuring the change of optical density at 400 nm. The solution of substrate was prepared by two steps. Firstly, 20 mg *p*-NPP was dissolved in 6 ml 2-propanol, then 100 mg gum-arabic and 207 mg sodium desoxycholate were dissolved in 90 ml buffer. 1 ml *p*-NPP solution was then added drop-wise to 19 ml buffer with dissolved reagents under constant vigorous stirring. The shelf life of prepared emulsion of substrate is 2 hours. The reaction mixture consisted of 1.8 ml substrate emulsion and 0.2 ml enzyme or buffer (in the case of control blank sample). The final concentration of substrate was 0.4 mM. Reaction conditions were as follows: pH 8.0, 40^{o}C, 3 - 5 min.

Quantitative determination of residual (during esterification) or released (during transesterification) **free fatty acids (FFA)** in reaction mixture was performed by titrimetric assay. Analysis was carried out by taking samples at definite time intervals and titrating them at 22^{o}C with sodium hydroxide solution in methanol (50 ± 5 mM) using phenolphthalein solution in ethanol as an indicator.

Thin-layer chromatography (TLC) and densitometry. The analysis of all reaction products was carried out by extracting 50 μl of reaction mixture aliquots at definite time intervals and analyzing by TLC. Samples were diluted with 50 μl diethyl ether (or 350 μl

when the reaction was solvent-free), mixed vigorously and kept at -20ºC until TLC analysis. TLC analysis was carried out on TLC silica gel G-25 plates (Merck) of different size (5x10 cm and 10x10 cm). The samples were applied to the marked start edge of a TLC plate (1.0 cm above the lower edge of the plate and 0.8 or 1.0 cm between each spot) using a specified TLC – a 5 µl Hamilton syringe. The volume of each sample for the experiments was 2 - 8 µl. After each sampling the syringe was washed with 2-propanol and n-hexane. The plate was then air-dried for 10 - 15 min before transferring it to the TLC tank containing a solvent system suitable for the separation of certain reaction products. The developed TLC plates were air-dried for about 10 - 15 min. The spots of unsaturated compounds were visualized using a saturated iodine chamber and in the case of saturated compounds the plates were sprayed with the mixture of methanol, sulphuric acid and acetic anhydride (20:2:2, v/v) and heated at 120^{o}C for 15 – 20 min.

Quantitative analysis (%) of reaction products separated by TLC (average of 3 - 4 assays) was performed with UVItec Cambrige Fire-reader imaging system and Uvitec Fire-reader 15.10 software by densitometry assessing the spot area and colour intensity.

The selection of appropriate solvent system for the separation of the products of different lipase-catalyzed reactions by TLC was carried out as described above, using the mixtures of investigated oils (0.4 g) in n-hexane (total volume 4.5 ml) diluted with diethyl ether (1:1, v/v).

Biodiesel synthesis

RO methanolysis. Reaction mixture, containing oil and methanol with a molar ratio of 1:2 – 1:16, respectively, water (0 – 40 %) and a lipase preparation (final concentration was 11 – 110 mg/ml which was converted to the units of lipase hydrolytic activity) in n-hexane, *t*-butanol or the mixture of these solvents, was incubated at 30^{o} – 50^{o}C for 1 – 168 h under continuous stirring. The enzyme-free reaction mixtures were incubated under the same conditions and used as control samples. The analysis of reaction products was carried out by TLC and densitometry, as described above.

RO transesterification with different acyl acceptors. Reaction mixture, containing oil and methanol, methyl or butyl acetate with a molar ratio of 1:4, respectively, a lipase preparation (final concentration was 44 mg/ml which was converted to the units of lipase hydrolytic activity) in n-hexane, *t*-butanol or solvent-free system, was incubated at room temperature (25^{o}C) for 30 min – 24 h under continuous stirring. The enzyme-free reaction mixtures were incubated under the same conditions and used as control samples. The analysis of reaction products was carried out by TLC and densitometry, as described above.

The effect of additives on biodiesel synthesis. Reaction mixture, containing oil and methanol with a molar ratio of 1:4, respectively, a lipase preparation and ectoine (final concentration was 1.1 mM) in n-hexane, *t*-butanol or solvent-free system, was incubated at 40^{o}C for 1 – 24 h under continuous stirring. The enzyme-free or ectoine-free reaction mixtures were incubated under the same conditions and used as control samples. The analysis of reaction products was carried out by TLC and densitometry, as described above.

The effect of organic solvent on biodiesel synthesis. Reaction mixture, containing RO and methanol with a molar ratio of 1:6, respectively, and a lipase preparation in various organic solvents, was incubated at 40^{o}C for 3 – 24 h under continuous stirring. Water from the reaction mixture was removed with molecular sieves (3 – 4 Å) or silica gel blue with

moisture indicator. The enzyme-free reaction mixtures were incubated under the same conditions and used as control samples. The analysis of reaction products was carried out by TLC and densitometry, as described above.

Synthesis of oleic acid (OA) and TMP esters – biolubricants - was carried out by three methods. The first set of reactions was conducted according to the method described by Uosukainen et al. [3]. Reaction mixture, containing methyl oleate (MO) or OA and TMP with a molar ratio of 3.5:1 and 4.5:1, respectively, water (15 %, w/w) and a lipase preparation (40 %, w/w), was incubated at 37^{o}C or 47^{o}C for 1 – 120 h under continuous stirring. During the second set of reactions water was replaced with *t*-butanol and the reactions were incubated at 60^{o}C. During the third set of reactions the amount of enzyme was reduced to 15 % (w/w) and reactions were carried out without any additional solvents. The enzyme-free reaction mixtures were incubated under the same conditions and used as control samples. The analysis of reaction products was carried out by TLC and densitometry, as described above.

Synthesis of fatty acids (FA) and β-citronellol esters. Reaction mixture, containing oil and alcohol with a molar ratio of 1:4, respectively, and a lipase preparation in various organic solvents, was incubated at 30^{o}C for 3 – 24 h under continuous stirring. The enzyme-free reaction mixtures were incubated under the same conditions and used as control samples. The analysis of reaction products was carried out by TLC and densitometry, as described above.

Esterification of organic acids with one or more carboxyl groups. Reaction mixture, containing acid and alcohol with a molar ratio of 1:1 (for monocarboxylic acids) or 1:2 (for dicarboxylic acids), respectively, and a lipase preparation in n-hexane, was incubated at 40^{o} – 60^{o}C for 1 – 144 h under continuous stirring. The enzyme-free reaction mixtures were incubated under the same conditions and used as control samples. The analysis of reaction products was carried out by TLC and densitometry as described above.

Transesterification and esterification of soap manufacturing wastes - suds. The mixture of suds was extracted with different organic solvents (1:1, v/v) as potential substrates for lipase-catalyzed reactions. Reaction mixture, containing 0.87 ml suds, a lipase preparation and 1.65 ml solvent, was incubated at 30^{o}C for 30 min – 5 h under continuous stirring. ***Part II.*** The mixture of suds was extracted with traditional organic solvents (1:1, v/v) used for lipase-catalyzed reactions. Reaction mixture, containing 0.87 ml suds, 65 – 195 µl methanol, a lipase preparation and 1.65 ml solvent, was incubated at 30^{o}C for 30 min – 48 h under continuous stirring. The enzyme-free reaction mixtures were incubated under the same conditions and used as control samples. The analysis of reaction products was carried out by TLC and densitometry as described above.

The cultivation of *Chlorella vulgaris* and *Scenedesmus dimorphus* microalgae strains in suds was carried out by standard microbiological methods [4].

All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

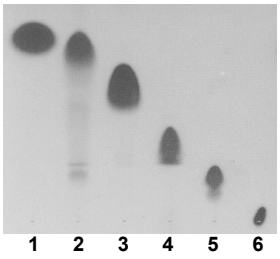


Fig.1. The TLC view of control samples.
1 - Methyl oleate;
2 - Trimethylolpropane trioleate;
3 – Triolein;
4 – Oleic acid;
5 – Diolein;
6 – Monoolein.

In this study, various lipase-catalyzed esterification and transesterification reactions were investigated. The optimal reaction conditions were determined using simple and accurate TLC and computer analysis methods that enable to follow the changes of all reaction mixture components simultaneously. For this reason, in order to apply the TLC method for the specific reactions, the selection of an appropriate elution system (mixture of organic solvents) was performed. Suitable elution system enables to separate all the components of the reaction effectively.

It was determined that the most suitable employing solvent system for our studied reactions is the one composed of light petroleum (b.p. 40 - 60^{o}C), diethyl ether and acetic acid with optimal volumetric ratio of 85:15:2, respectively (Fig. 1).

Biodiesel synthesis (RO methanolysis)

It is well known that during lipase-catalyzed oil methanolysis one of the main problems is possible enzyme inactivation by alcohol. Although the mechanism of this inactivation is not completely resolved, it is proposed that due to its insolubility in oils methanol forms a new liquid phase which inactivates lipases by denaturating them [5-7]. Moreover, the hydrophylic glycerol, that forms during the reaction, is also insoluble in oils, for this reason it can easily adsorb to the surface of immobilized lipase which has also a negative effect on the stability and activity of enzyme [8, 9]. Using various hydrophobic organic solvents such as n-hexane for lipase-catalyzed reactions does not solve the above mentioned problem because of the poor solubility of methanol and glycerol [10, 11]. One of the solutions to avoid this inactivation is to use a suitable organic solvent. Thus, in this study the reactions were carried out not only in n-hexane as a commonly used organic solvent for lipase-catalyzed reactions but also in *t*-butanol (tertiary alcohols are poor substrates for lipases) in which dissolves all the reaction mixture components forming a homogeneous medium. It is stated that using *t*-butanol can greatly improve the yields of the products obtained during lipase-catalyzed processes [8, 12-15]. Firstly, it was investigated the effect of the solvent employed on the progress of Lipoprime 50T lipase-catalyzed RO methanolysis reaction (Fig. 2).

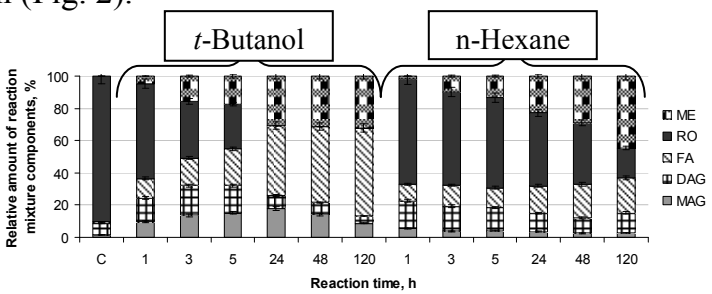


Fig. 2. The effect of solvent on the progress of Lipoprime 50T lipase-catalyzed RO methanolysis reaction (molar ratio of oil to methanol was 1:4). C – RO. ME – methyl oleate, RO – rapeseed oil, FA – fatty acids, DAG – diacylglycerols, MAG – monoacylglycerols.

The highest methyl esters (ME) yield of 45 % was obtained in n-hexane. Although after 120 h the yield of ME was lower in *t*-butanol in comparison to n-hexane, the hydrolysis of RO was very fast in this case (no RO left after 24 h from the beginning of the reaction). Thus, the hydrolysis was more favorable in *t*-butanol, while transesterification was more effective in n-hexane. Another important variable affecting the yield of biodiesel is the molar ratio of oil to alcohol. In order to shift the methanolysis reaction in toward direction, it is necessary to use an excess amount of methanol as methanolysis is a reversible reaction. Biodiesel production usually increases with increasing methanol to oil ratio of 3:1 and then decreases because of the lipase inactivation by insoluble excess of short chain alcohol [5-7]. The molar ratios of oil to methanol ranged from 1:2 to 1:8 were studied to evaluate the effect of methanol on lipase activity in *t*-butanol. The effect of temperature (30, 40 and 50°C) was also investigated (Fig. 3).

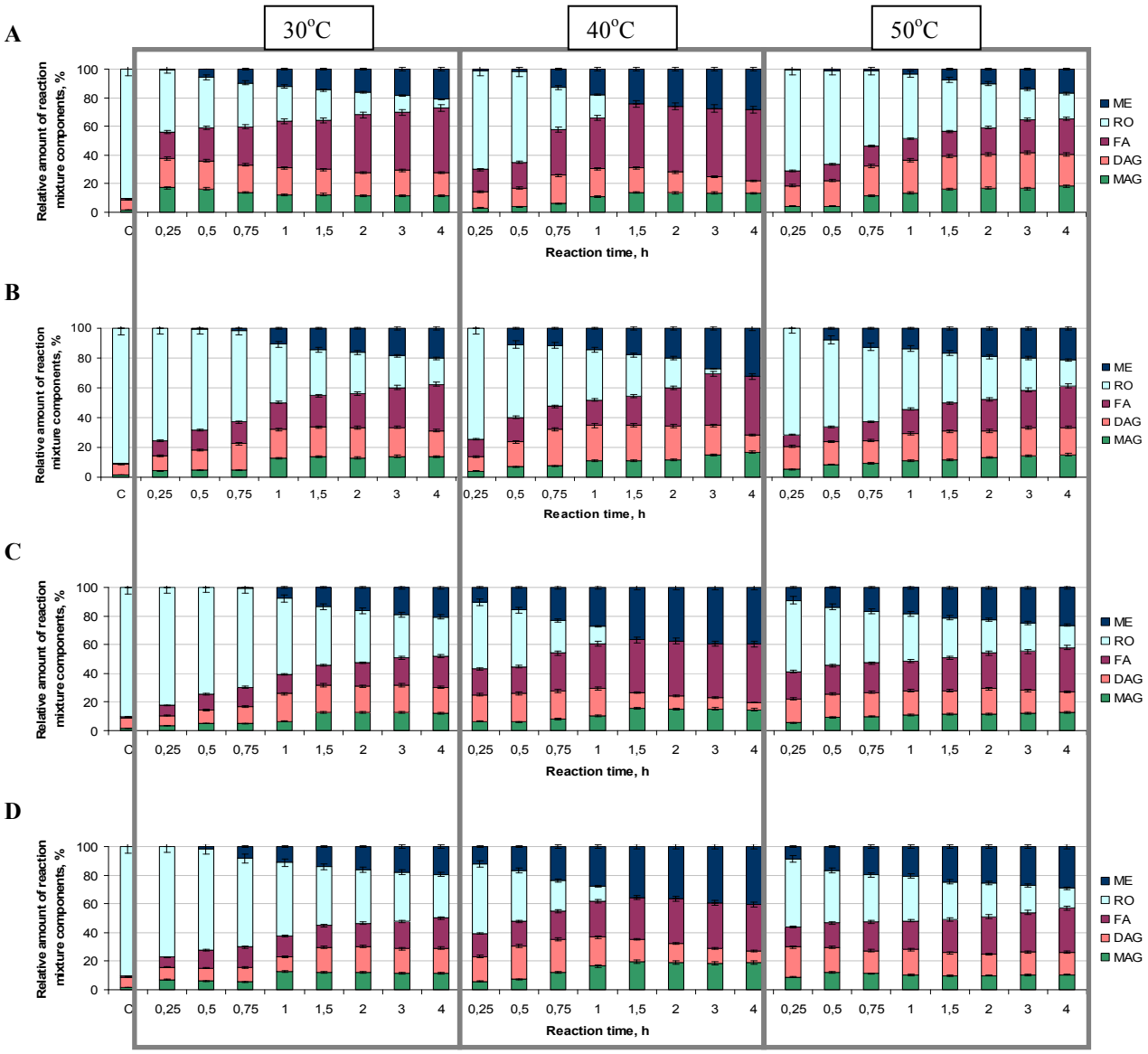


Fig. 3. The effect of molar ratio of substrates, reaction temperature and time on productivity of Lipoprime 50T-catalyzed RO methanolysis in *t*-butanol. Molar ratio of RO to methanol: A – 1:2, B – 1:4, C – 1:6, D - 1:8. Symbols are the same as in Fig. 2.

When the molar ratio of oil to methanol was in range of 1:6 – 1:8, high conversions were achieved and no significant differences were detected with different oil to methanol molar ratios within this range, indicating that under studied conditions Lipoprime 50T was tolerant to methanol presence. 40°C was determined to be an optimal temperature. Above 40°C, the process effectiveness decreased, possibly owing to some enzymatic deactivation. Considering these results, the reaction temperature of 40°C, the molar ratio of RO to methanol 1:6 – 1:8 and the reaction duration of 1.5 h were selected as the optimal conditions for the reaction in *t*-butanol (Fig. 3).

Since the reaction manner was dramatically different in both studied organic solvents (Fig. 2), analogous reactions were carried out in n-hexane (Fig. 4). As the processes investigated in n-hexane were relatively slow, the reaction duration of 48 h was chosen for the experiments.

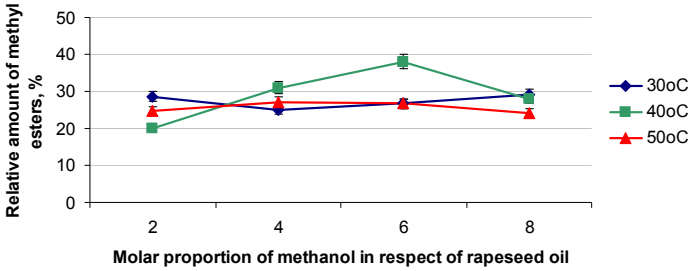


Fig. 4. The effect of molar ratio of substrates and reaction temperature on productivity of Lipoprime 50T-catalyzed RO methanolysis in n-hexane.

The optimal reaction conditions in n-hexane were the same as in *t*-butanol: the highest yield of ME was obtained at 40°C when the molar ratio of oil to methanol was 1:6. Analogous reactions were carried out in the mixtures of n-hexane and *t*-butanol, but no significant increase in the yield of the products was observed.

The effect of water content. Water content is known to be one of the main factors that affect the activity of lipase in non-aqueous medium. A small amount of water is necessary to keep the active conformation of the enzyme. However, excessive water promotes the hydrolysis of substrate and decrease the yield of the products. To obtain high RO conversions, the aqueous phase should be reduced to avoid ME hydrolysis and aggregation of enzyme molecules. On the other hand, the water content must be large enough to prevent enzyme inactivation by the methanol [6, 16-20].

The effect of water content on the lipase-catalyzed methanolysis of RO was investigated via the addition of a definite quantity of water in a range from 0 to 40 % (v/v) to the reaction mixture consisting of methanol and RO as substrates, *t*-butanol or n- hexane as a solvent, and Lipoprime 50T as the enzyme. The reaction conditions were as follows: the molar ratio of RO to methanol 1:6, 40°C, 24 h (Fig. 5).

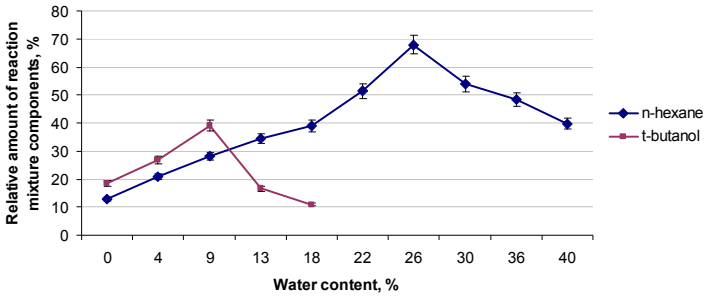


Fig. 5. The effect of water content on productivity of Lipoprime 50T-catalyzed RO methanolysis.

The RO methanolysis in both solvents had a clear water content dependence. The ME yield increased with increasing the water content from 0 to 9 % (v/v) in *t*-butanol and from 0 to 26 % (v/v) in n-hexane. The product yield sharply decreased when water content was above these optimal values. This is because a higher water content shifts the reaction toward hydrolysis of substrate rather than the synthesis of ester. Under optimal conditions the highest ME yields of 38 % and 68 % were obtained in *t*-butanol and n-hexane, respectively. These results support widely discussed dependence of organic solvent used for the transesterification reaction and water content required for maximum conversion. The water content required for high yields of the product increases with increasing the reaction medium hydrophobicity [16].

The effect of molar ratio of substrates and lipase dosage. When the reactions were carried out in n-hexane with a water content of 26 %, the highest ME yield of about 68 % was obtained. Therefore, new methanolysis reactions were carried out to determine the optimal molar ratio of substrates in n-hexane in order to obtain even higher yield of the desired product (Fig. 6 A). The molar ratio of RO to methanol ranged from 1:2 to 1:16 was studied to evaluate the effect of methanol on lipase activity in n-hexane.

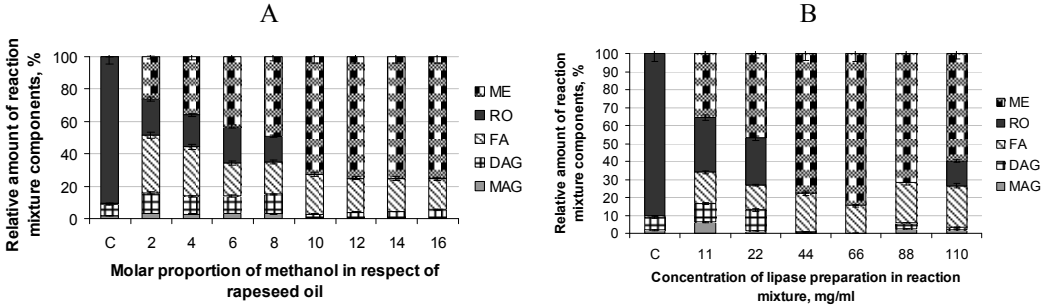


Fig. 6. The effect of RO to methanol molar ratio (A) and a lipase dosage (B) on productivity of Lipoprime 50T-catalyzed RO methanolysis in n-hexane. Reactions were performed for 24 h at 40°C with water content of 26 % (v/v). A: Amount of enzyme preparation was 44 mg/ml. B: molar ratio of RO to methanol was 1:16. Symbols are the same as in Fig. 2.

When the molar ratio of oil to methanol was in range of 1:12 – 1:16, ME yields of around 75 % were achieved after 24 h, and no significant differences were detected with different oil to methanol molar ratios within this range, indicating that Lipoprime 50T was tolerant to methanol presence under studied conditions. Considering earlier determined optimal conditions, the effect of Lipoprime 50T lipase preparation quantity on methanolysis of RO in n-hexane has been evaluated (Fig. 6 B). The ME yield increased with increasing

the lipase dosage from 11 to 66 mg/ml. When the lipase dosage reached 66 mg/ml, the ME yield of around 85 % was obtained within 24 h. However, further increase in lipase dosage above 66 mg/ml was not capable of enhancing the ME yield, possibly owing to some enzymatic deactivation because of the formation of enzyme excess agglomerates.

Effect of methyl and butyl acetates as acyl acceptors. Some studies have shown that using methyl, ethyl or butyl acetates as acyl acceptors for biodiesel production significantly enhances the stability of lipase. Acetate, instead of alcohol, and the by-product triacetylglycerol, instead of glycerol, has no negative effect on the activity of lipase [21, 22]. For this reason the productivity of Lipolase 100L-catalyzed RO transesterification reaction with methanol, methyl and butyl acetate was compared. In order to evaluate the maximum economical potential of the process, reactions were carried out under exceptionally mild conditions - reaction temperature was 25°C. The reactions were conducted in two different solvents - *t*-butanol and n-hexane – and compared with solvent-free processes (Fig. 7).

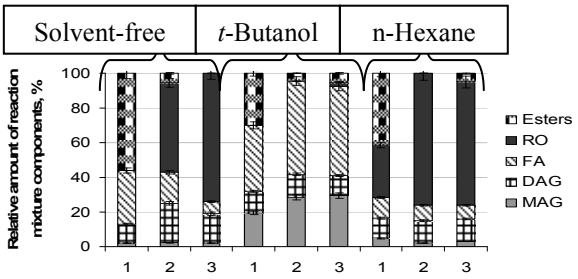


Fig. 7. The effect of acyl acceptor (1- methanol, 2 – methyl acetate, 3 – butyl acetate) and solvent on productivity of Lipolase 100L-catalyzed RO transesterification. Reaction conditions: molar ratio of oil to acyl acceptor was 1:4, 24 h. Symbols are the same as in Fig. 2.

RO transesterification with traditional acyl acceptor - methanol - was far more efficient than the reactions with acetates (the highest yield of esters was only 5 %). Therefore, it is clear that under studied conditions the use of acetates instead of methanol is not an effective way of increasing reaction productivity.

Effect of ectoine. In order to improve the productivity of lipase-catalyzed oil methanolysis various additives can be used. It was shown that the addition of compatible solutes improves the yield of biodiesel by increasing enzymatic activity. For example, when the cyclic amino acid ectoine ((S)-2-methyl-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid, chemical structure shown in Fig. 8, [23]), one of the representative compatible solutes with the characteristic of zwitterions, was added to the reaction mixture, the yield of biodiesel increased by 20.9 % compared to the yields obtained without this additive. Ectoine acts as a stabilizer of proteins, nucleic acids and cells against some adverse conditions. It was shown that the supplementation with an appropriate amount of ectoine leads to a lower affinity of the lipase for methanol and a higher affinity for TAG. Thus, the addition of ectoine could be a new method for improving the productivity of lipase-catalyzed oil methanolysis by reducing a negative effect of methanol. [23].

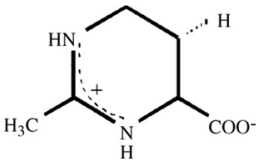


Fig. 8. Chemical structure of ectoine ((S)-2-methyl-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid) [23].

Six liquid (Lipolase 100L, Lipex 100L, Lipozyme TL100L, Resinase A2X, Palatase 20000L, Lecitase Ultra) and seven immobilized (Lipoprime 50T, Lipolase 100T, Lipex

100T, Lipozyme TL IM, Lipozyme RM IM, Novozym 435, Lipoclean 2000T) commercial enzymes were chosen to investigate the effect of ectoine on productivity of RO methanolysis. Reactions were carried out in n-hexane (Fig. 9 A), *t*-butanol (Fig. 9 B) and solvent-free system (Fig. 9 C).

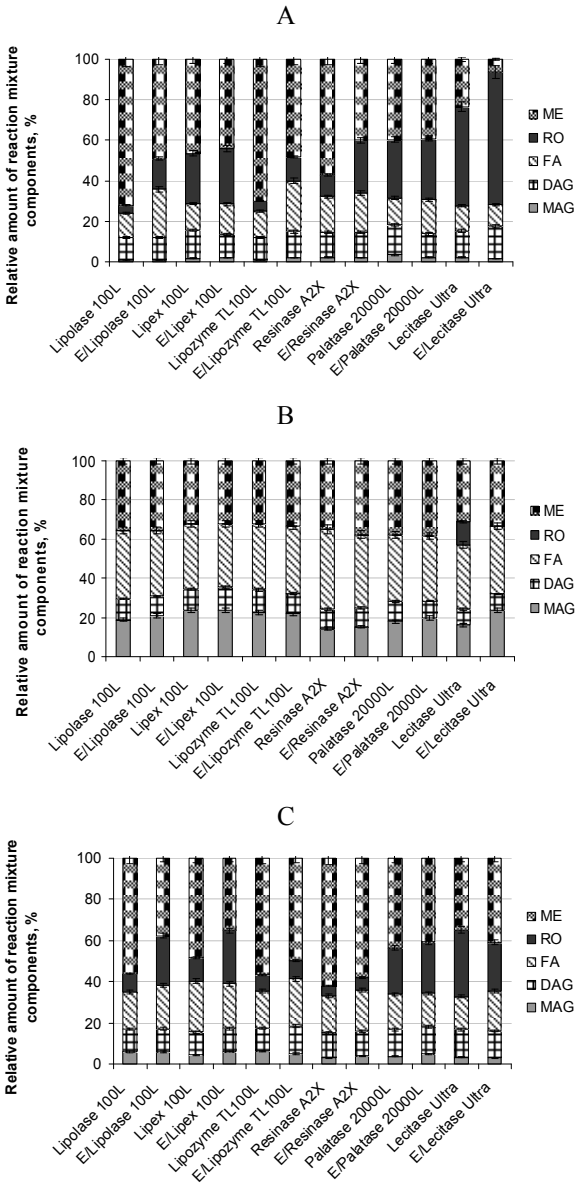


Fig. 8. The effect of ectoine on productivity of lipases-catalyzed RO methanolysis in n-hexane (A), *t*-butanol (B) and solvent-free system (C). Reaction conditions: 40°C, molar ratio of RO to methanol 1:6, 24 h. Reaction mixtures with ectoine are marked with „E/". Symbols are the same as in Fig. 2.

Surprisingly, the ectoine had a negative or no effect on most lipase-catalyzed reactions under studied conditions. The ME yield obtained in n-hexane decreased by 22 - 23 % when the reaction was catalyzed by Lipolase 100L or Lipozyme TL100L, and by 17 % when Resinase A2X or Lecitase Ultra was used. In other cases the negative effect of ectoine was insignificant, the ME yield decreased by 1 - 4 % compared to the amounts obtained with no additives (Fig. 8 A). Ectoine had no effect on the reactions carried out in *t*-butanol

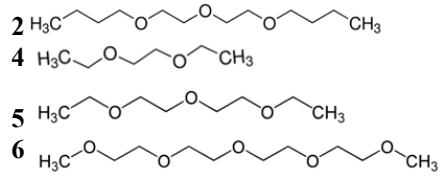
in all cases, except for Lecitase Ultra lipase-catalyzed reaction (the ME yield increased only slightly (by 3 %) but all RO was hydrolyzed compared to the reaction without ectoine when 12 % RO was left) (Fig. 8 B). Ectoine had a negative effect on the reactions conducted in solvent-free systems except for Lecitase Ultra lipase-catalyzed methanolysis (slightly increased ME yield was observed) (Fig. 8 C). Ectoine had no effect on reactions catalyzed by immobilized enzymes under studied conditions. For this reason the results are not shown.

Thus, it is clear that under studied conditions supplementation with ectoine is not an effective way of increasing reaction productivity. The amount of ectoine (1.1 mM) was chosen according to the data found in the literature, for this reason it is likely that in our case it was not an optimal ectoine concentration since it has been reported that an excessive quantity of ectoine can inhibit the enzymatic activity [23].

Effect of organic solvents. Diethers of glycol (glymes) are saturated polyethers containing no other functional groups. As compared to glycols (such as polyethylene glycols), glymes do not carry free hydroxyl groups and thus are chemically inert compounds. Most glymes are completely miscible with both organic solvents and water, thus, all substrates of different origin (hydrophobic and hydrophilic) are soluble in these solvents, forming a homogeneous reaction mixture. Moreover, glymes possess other valuable properties such as low viscosity, high chemical and thermal stability, low toxicity and good biodegradability. It was reported that glymes can lead to higher enzyme activity and stability than *t*-butanol or ionic liquids. In the presence of glymes some lipases show a very high tolerance to high methanol concentrations. Thus, these compounds could be environmentally friendly and relatively inexpensive solvents for lipase-catalyzed reactions such as the enzymatic production of biodiesel [24]. According to the data presented in the literature four the most suitable glymes (Table 1) were chosen for RO methanolysis catalyzed by different lipases [24]. Analogous reactions were carried out in *t*-butanol and n-hexane as well. Six liquid (Lipolase 100L, Lipozyme TL100L, Lipex 100L, Resinase A2X, Palatase 20000L, Lecitase Ultra) and two immobilized (Novozym 435 ir Lipoclean 2000T) commercial enzymes were chosen for the experiments (Fig. 9)

Table 1. Organic solvents used for investigation

	Solvent	logP
1	n-Hexane	+3.50
2	Diethylene glycol dibutyl ether	+2.24
3	*tert*-Butanol	+0.83
4	Ethylene glycol diethyl ether	+0.75
5	Diethylene glycol diethyl ether	+0.27
6	Tetraethylene glycol dimethyl ether	-1.26



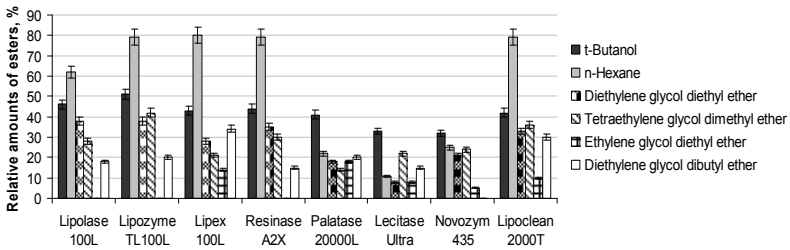


Fig 9. The effect of solvent on productivity of lipase-catalyzed RO methanolysis after 24 h.

The highest ME yields of about 80 % were obtained in n-hexane with Lipozyme TL100L, Lipex 100L, Resinase A2X and Lipoclean 2000T. Reactions catalyzed by Palatase 20000L, Lecitase Ultra and Novozym 435 were more effective in *t*-butanol compared to other solvents. The ME yields obtained in glymes were significantly lower as compared to traditional commonly used solvents – *t*-butanol and n-hexane.

As it was mentioned earlier, water content is known to be one of the main factors that affect the activity of lipase in non-aqueous medium. In order to obtain higher ME yields the effect of water content on activity of immobilized lipases was investigated. Reactions were carried out without additional water quantity and compared with the results obtained during previous experiments (Fig. 10).

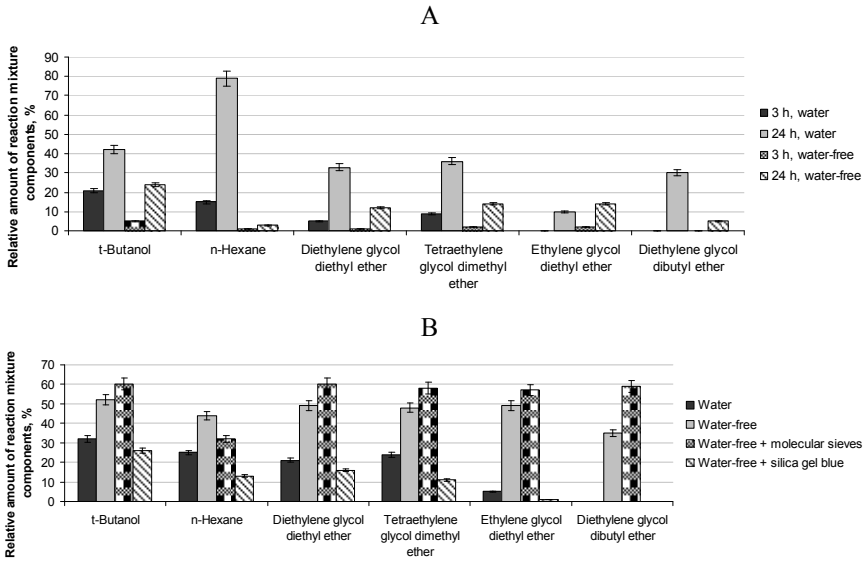


Fig. 10. The effect of water on productivity of Lipoclean 2000T (A) and Novozym 435 (B) lipases-catalyzed RO methanolysis after 24 h.

Non-aqueous conditions had a significant negative effect on activity of Lipoclean 2000T with the exception of ethylene glycol diethyl ether (slightly increased ME yield was observed). The highest negative impact was observed in n-hexane where the ME yield reached about 80 % after 24 h during the reaction with water, while only 5 % of ME were obtained during the reaction under non-aqueous conditions (Fig. 10 A).

The effect of non-aqueous medium on Novozym 435 lipase-catalyzed reaction was the opposite (Fig. 10 B). A significant increase in ME yield was observed in non-aqueous medium. Moreover, when water removing agents such as molecular sieves were applied, even higher ME yields were obtained (with the exception of n-hexane). When silica gel blue was added to the reaction mixture, the reaction productivity decreased dramatically possibly due to mechanical grating of lipase molecules by silica gel grains because of the mixing with magnetic stirrer. Thus, molecular sieves are a great tool to remove water from the reaction mixture in order to obtain higher yields of desired products.

Synthesis of biolubricants: OA esterification and MO transesterification with TMP

An increasing environmental awareness in society has given rise to intensified efforts to bring lubricants derived from renewable resources (biolubricants) into the market due to

their biodegradability and environmentally benign nature. In addition, such lubricants exhibit better lubricity, an excellent viscosity index, and lower volatility than petroleum-based analogs. Despite these advantages, vegetable oil based lubricants have been slow in gaining wide acceptance as lubricants due to a poor ageing stability of vegetable oils as they are composed of some polyunsaturated FA which are responsible for the poor oxidation stability [25]. For this reason, preference is being given to the use of synthetic esters composed of OA and polyhydric alcohol as the base fluids for industrial applications. Common fat-based lubricants are of the following types: branched polyol esters, mono- and dibasic esters, and glycol esters. The most important group is the polyol esters derived from branched polyol. The absence of a hydrogen atom on the β-carbon of its structure ensures a high thermal stability of these esters, a feature rare in vegetable oils. A combination of selected FA or FA esters with alcohol can produce synthetic esters with an appropriate structure for various applications. The preference is being given to a lipase-catalyzed synthesis as an environmentally acceptable alternative to chemical methods [3, 25].

Trimethylolpropane (2-(hydroxymethyl)-2-ethylpropane-1,3-diol) was used for the synthesis of the esters. The synthesis of TMP esters was performed by MO transesterification and OA esterification, using the commercial lipases as a biocatalyst (esterification reaction scheme is shown in Fig. 10, [26]). The products of the reactions are TMP esters which can be used as raw materials for a biodegradable hydraulic fluid and other lubricants. The conversions were obtained at ambient pressure, while many other processes described in the literature are conducted under vacuum at different pressures [3].

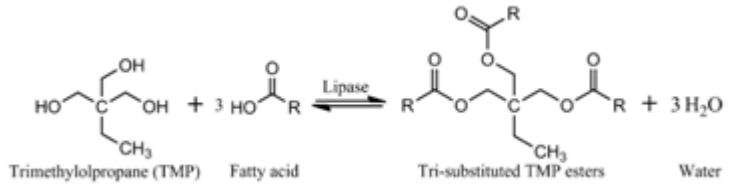


Fig. 10. The reaction scheme of lipase-catalyzed fatty acid esterification with TMP [26].

Preliminary experiments with Lipoprime 50T lipase clearly suggested that the conversion of MO or OA to desired TMP esters increased with increasing the temperature. For this reason, the temperature of 60°C was used for the experiments. Considering the fact that reaction medium is a very important parameter for the effectiveness of lipase-catalyzed processes, the reactions in two different media were compared. Aqueous solution (Fig. 11 B and Fig. 12 B) was used as a reaction medium to ensure an environmentally friendly process and *t*-butanol (Fig. 11 A and Fig. 12 A) was chosen as it had been reported to be a good solvent for lipase-catalyzed reactions due to its ability to dissolve all reaction mixture components of different origin. Experiments were carried out with MO (Fig. 11) or OA (Fig. 12) and TMP in order to compare the effectiveness of both reactions.

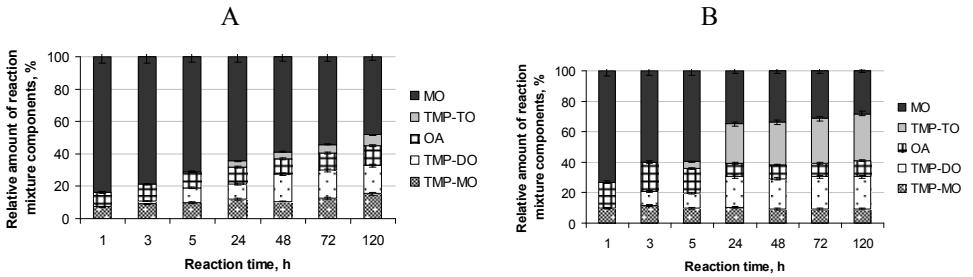


Fig. 11. Time course of Lipoprime 50T-catalyzed MO transesterification with TMP in *t*-butanol (A) and aqueous solution (B). MO – methyl oleate, TMP-TO – trimethylolpropane trioleate, OA – oleic acid, TMP-DO – trimethylolpropane dioleate, TMP-MO – trimethylolpropane monooleate.

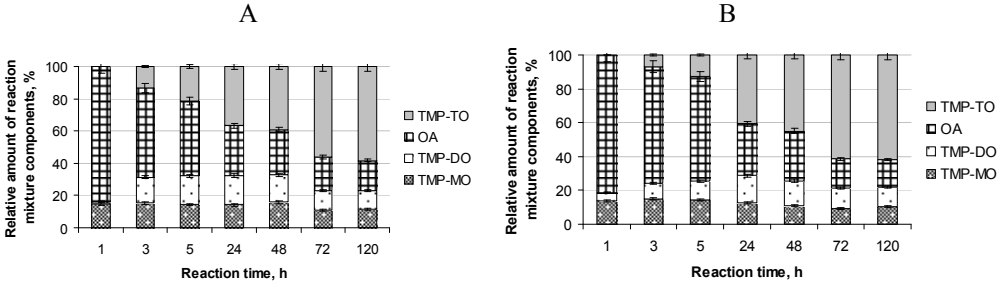


Fig. 12. Time course of Lipoprime 50T-catalyzed OA esterification with TMP in *t*-butanol (A) and aqueous solution (B). Symbols are the same as in Fig. 11.

Both lipase-catalyzed processes in both solvents were clearly time-dependent. Reactions were more efficient in aqueous solution (Fig. 11 B and Fig. 12 B) than in *t*-butanol (Fig. 11 A and Fig. 12 A). The maximum total conversion to TMP mono-, di- and trioleates of around 83 % and to TMP-TO of about 62 % was obtained in aqueous solution within 72 h of the esterification reaction (Fig. 12 B). Although the yields are not high enough for industrial application, the process shows its potential to be optimized in near future as the reactions were carried out at ambient pressure, while most other processes described in the literature are conducted under vacuum at a certain value of pressure.

In order to find the most suitable lipase for the studied reaction, the activity of ten different commercial enzymes was compared. The reactions were conducted in aqueous (Fig. 13 A) or non-aqueous (Fig. 13 B) medium and the dosage of lipase preparation was reduced from 40 to 15 % (w/w).

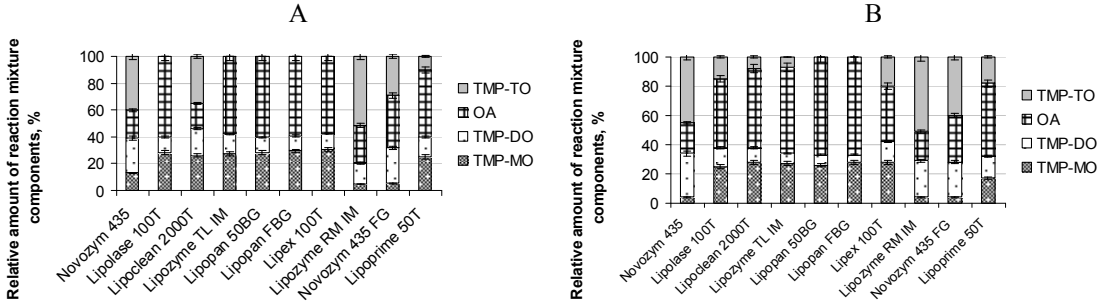


Fig. 13. Productivity of OR esterification with TMP within 72h in aqueous (A) and non-aqueous (B) medium catalyzed by different commercial enzymes. Symbols are the same as in Fig. 11.

The reduction of lipase dosage had a drastical negative effect on Lipoprime 50T-catalyzed reaction: TMP-TO yield decreased from 62 to 10 %. Moreover, no TMP-TO was

synthesized when Lipolase 100T, Lipozyme TL IM, Lipopan 50BG, Lipopan FBG or Lipex 100T was used. The highest TMP-TO yield of about 52 % was obtained with Lipozyme RM IM lipase (Fig. 13 A). Non-aqueous conditions had no effect on the highest ME yield obtained with Lipozyme RM IM and on the processes catalyzed by Lipopan FBG and Lipopan 50BG. Non-aqueous conditions had negative effect only on Lipoclean 2000T lipase-catalyzed reaction – the yield of TMP-TO decreased from 35 to 8 %. The positive effect was observed with all other enzymes (Fig. 13 B). The highest TMP-TO yields under non-aqueous conditions were obtained with Lipozyme RM IM and Novozym 435. For this reason the time course of these reactions was investigated in more detail (Fig. 14).

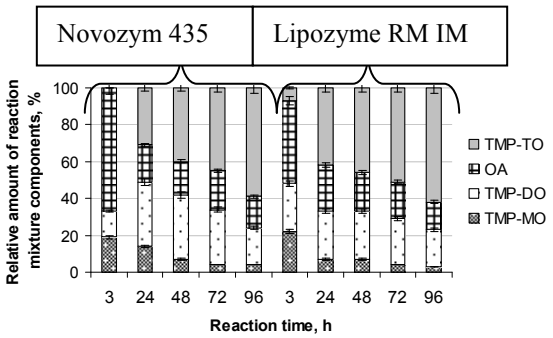


Fig. 14. Time course of lipases- catalyzed OR esterification with TMP under non-aqueous conditions. Symbols are the same as in Fig. 11.

The highest TMP-TO yields of 59 and 62 % (the maximum total conversion to TMP mono-, di- and trioleates of 83 and 85 %) were obtained with Novozym 435 and Lipozyme RM IM lipases, respectively, after 96 h from the reaction beginning (Fig 14).

Transesterification of various oils with terpenols

Esters of terpene alcohols are very important flavour and fragrance compounds widely used in food, beverage, cosmetics and pharmaceutical industries. Enzymatic synthesis of such compounds is beneficial not only from an environmental or energy-saving point of view but also for product quality which is often governed by operating temperature [27].

The selection of new substrates suitable for the reactions catalyzed by commercial lipases was carried out. It was determined that terpenols such as geraniol and β-citronellol are perfect acyl acceptors for oils transesterification reaction. For this reason, transesterification of 11 different oils (RO - rapeseed, FFO - false flax, SnO - sunflower, OO - olive, CsO - castor, CB - cocoa butter, LSO - linseed, RBO - rice bran, CcO - coconut, GSO - grape seed, SfO - safflower) with one of the terpenols - β-citronellol - in *t*-butanol and n-hexane catalyzed by two commercial enzymes - Lipolase 100L and Lipex 100L – was compared (Fig. 15).

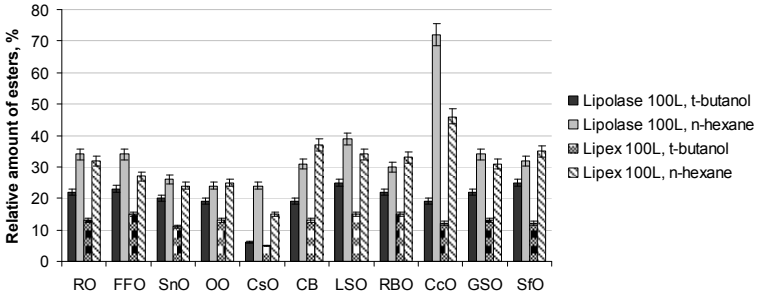


Fig. 15. The effect of solvent, lipase and oil on the productivity of transesterification with β-citronellol within 24 h.

Lipolase 100L was determined to be more active than Lipex 100L under studied conditions. Both enzymes were more active in n-hexane as compared to *t*-butanol. The highest esters yield of 72 % was obtained with coconut oil when Lipolase 100L was used. For this reason this oil was chosen for further experiments. CcO contains more than 90 % of saturated FA (in the form of TAG), most of them are lower chain saturated FA (lauric acid, C12:0). Cocoa butter is also composed mainly of saturated FA but most of them are longer chain FA - palmitic (C16:0) and stearic (C18:0). Thus, it could be preliminary assumed that our studied enzymes are more specific for shorter chain saturated FA. However, it should be noted that CcO is more soluble in alcohols than other oils and this could also be the reason for the higher yields of esters obtained.

In order to choose the best enzyme for the coconut oil transesterification with β-citronellol in n-hexane the selection of suitable enzyme was performed. The activity of 15 different commercial enzymes was compared (Fig. 16).

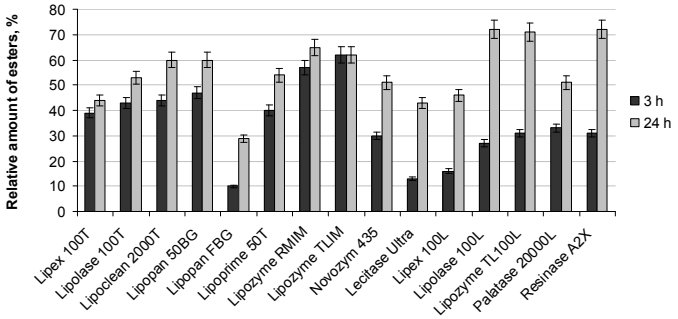


Fig. 16. The effect of enzyme and reaction duration on coconut oil transesterification with β-citronellol.

Lipolase 100L, Lipozyme TL100L and Resinase A2X were determined to be the best enzymes for the studied reaction – the yield of esters was higher than 70 % after 24 h. As previously studied Lipolase 100L lipase was one of the most active enzymes, it was chosen for further experiments. As the reaction manner was drastically different in n-hexane and *t*-butanol, new reactions in 20 different solvents (Table 2) were carried out in order to determine the effect of organic solvent on activity of the enzyme (Fig. 17).

Table 2. Organic solvents used for the investigation

	Solvent	LogP		Solvent	LogP
1	n-Octane	+4.78	11	*t*-Butylmethyl ether	+0.94
2	Isooctane	+4.37	12	*t*-Butanol	+0.83
3	n-Heptane	+4.00	13	Pyridine	+0.62
4	Cyclohexane	+3.80	14	2-Butanone	+0.61
5	n-Hexane	+3.50	15	Tetrahydrofuran	+0.50
6	n-Pentane	+3.25	16	Acetone	-0.26
7	Toluene	+2.50	17	Acetonitrile	-0.36
8	Benzene	+2.00	18	Dimethylformamide	-0.83
9	Diisopropyl ether	+2.00	19	Dioxane	-1.10
10	*t*-Amyl alcohol	+1.30	20	Dimethyl sulfoxide	-1.30

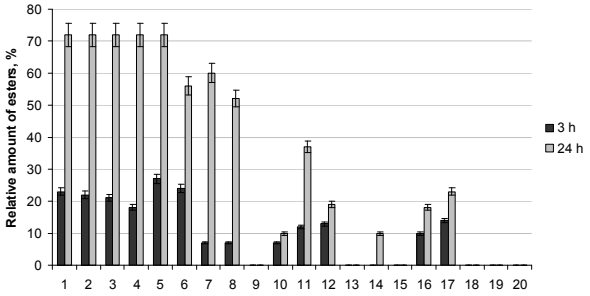


Fig. 17. The effect of organic solvent and reaction duration on productivity of Lipolase 100L lipase-catalyzed coconut oil transesterification with β-citronellol. The numbers correspond to the numbers of solvents in Table 2.

The reaction was inhibited in diisopropyl ether, pyridine, tetrahydrofuran, dimethylformamide, dioxane and dimethyl sulfoxide and no logP dependence was observed. However, Lipolase 100L showed the highest activity in the solvents with logP values higher or equal to +3.50: n-octane, isooctane, n-heptane, cyclohexane and n-hexane (Fig. 17, 1 - 5 columns) – ester yields of about 72 % were obtained. These results support widely discussed dependence of lipase activity and logP value of organic solvent used for the transesterification reaction. The higher activity of enzymes are observed in the solvents with logP values higher than 2. Moreover, it was shown that enzymes are more stable in more hydrophobic environment [28].

Esterification of carboxylic acids containing one or more carboxyl and/or hydroxyl groups

Substrate specificity of lipases is often investigated using alcohols with different number of hydroxyl groups (e.g. diols, triols). However, it is difficult to find any informative data about enzymatic esterification of carboxylic acids containing more than one carboxyl group. The main methods are based on chemical techniques or patented. For this reason five different carboxylic acids (Fig. 18) were chosen in order to investigate lipase specificity upon these compounds. Novozym 435 lipase was chosen due to its wide substrate specificity described in literature. The esterification of these acids were carried out with two different alcohols – β-citronellol and oleyl alcohol. The chromatographic view of the products, synthesized during the Novozym 435 lipase-catalyzed esterification with β-citronellol reaction is shown in Fig. 19.

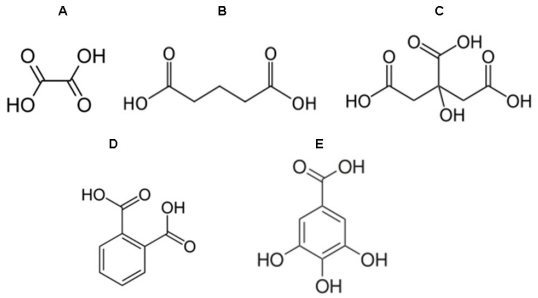


Fig. 18. Chemical structure of carboxylic acids used for the investigation: A – oxalic; B – glutaric; C – citric; D – ftalic; E – gallic.

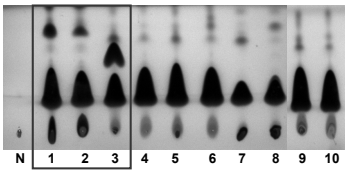


Fig. 19. The chromatographic view of the products generated after 24 h during Novozym 435 lipase-catalyzed carboxylic acids (1 – oxalic, 3 – glutaric, 5 – ftalic, 7 – gallic, 9 – citric) esterification with β-citronellol. Controls: N – Enzyme with solvent; Reaction mixtures without enzyme: 2 – oxalic; 4 – glutaric; 6 – ftalic; 8 – gallic; 10 – citric.

The reaction was productive only with oxalic and glutaric acids (Fig. 19, 1 and 3 lanes). Enzyme showed no activity with other carboxylic acids possibly due to some steric effects of these compounds (Fig. 18, C-E) [29, 30]. The reaction with oxalic acid was productive without enzyme as well (Fig. 19, 2 lane). There are some data in literature about the spontaneous esterification of oxalic acid under some aggressive conditions such as high temperature, however, our reactions were conducted under mild conditions that are not favorable for spontaneous processes, for this reason the reaction requires a more detailed examination with other methods. Moreover, when the oxalic acid esterification was carried with two different alcohols – β-citronellol and oleyl alcohol – and two enzymes – Novozym 435 and Lipozyme LT100L some more interesting results were obtained (Fig. 20).

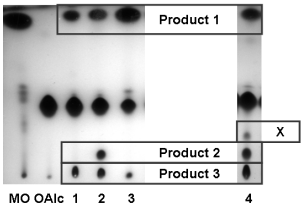


Fig. 20. The chromatographic view of the products generated during Novozym 435 (1) and Lipozyme TL100L (2)-catalyzed oxalic acid esterification with oleyl alcohol and Lipozyme TL100L-catalyzed oxalic acid esterification with β-citronellol (4) reactions. Controls: MO – methyl oleate; OAlc – oleyl alcohol. 3 – reaction mixture without enzyme.

Four different products were produced during oxalic acid esterification under different conditions (Fig. 20). However, the product identification was impossible because of the lack of control samples that could enable comparative analysis by TLC. For this reason the mass spectrometry and chemical analysis of these compounds will be performed by colleagues from the Faculty of Chemistry (Department of Organic Chemistry, Vilnius University) in near future. The analysis of the product generated during Novozym 435 lipase-catalyzed glutaric acid esterification with β-citronellol was already performed. The

results confirmed that glutaric acid and β-citronellol diester (chemical structure is shown in Fig. 21 B) is produced during the reaction.

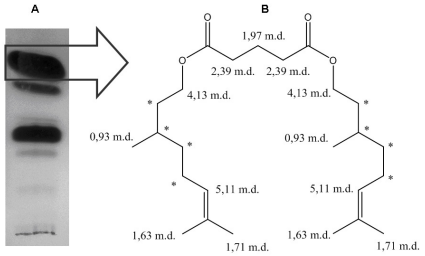


Fig. 21. The chromatographic view of the products generated during Novozym 435-catalyzed glutaric acid esterification with β-citronellol (A) and the structure with chemical shifts of hydrogens in ^{1}H BMR spectrum of one of the products - glutaric acid and β-cytronellol diester (doc. V. Masevičius) (B).

Investigation of soap manufacturing wastes – suds – utilization capabilities during transesterification/esterification processes catalyzed by lipases

The utilization and/or recycling of fatty wastes accumulating during various industrial processes is a huge worldwide problem. Wastes generated during the soap manufacturing process (suds) are generally used for the production of a lower quality technical soap (laundry soap) or washing and cleaning products such as scouring pastes. There is another possible way of utilization of these wastes - suds can be a potential substrate of lipases for the production of various FA esters (e.g. biodiesel) by transesterification/esterification of suds with different alcohols. As it is known, the most important reason for the slow progress of the process of commercialization of biodiesel is a high production cost. 70 – 80 % of this cost depends on the price of the raw material (edible oil) used for the production [20, 31]. It is estimated that the use of fatty wastes instead of pure oil could reduce the cost of biodiesel by 60 – 90 % [32].

The composition of fatty wastes is quite different from the commonly used oils. Fatty wastes usually have a significantly higher content of water and free FA. For this reason traditional chemical catalysis cannot be applied due to the formation of soaps. Thus, the most appropriate methods are based on enzymatic catalysis as free FA can be esterified by lipases [33]. Another raw material for the production of biodiesel is algae. Some algae species accumulate extremely high amounts of lipids, that are similar in its composition to traditional vegetable oils [34, 35]. Algae are microscopic autotrophic and heterotrophic organisms that are able to grow and multiply really fast in both synthetic as well as natural environments (e.g. effluent) [36]. For this reason suds possibly could be used as a medium suitable for the growth of algae.

Thanks to *de minimis* aid (innovation voucher, the recipient was JSC „NAUJOJI RINGUVA“) the research „Perspectives of biotechnological utilization of the products generated during lipolytic enzymes catalyzed reactions when one of the substrates are soap manufacturing wastes – suds“ was performed.

Firstly, the composition of the suds was analyzed. Composition of chemical elements was determined by atomic absorption spectrometry by prof. S. Tautkus (Faculty of Chemistry, VU) and the analysis of fatty components was performed by gas chromatography by Dr. G. Švirmickienė (Lithuanian University of Health Sciences,Veterinary Academy, Institute of Animal Science). The main chemical elements

found in suds were calcium, iron, sodium and magnesium, a slight amount of potassium and lead traces were detected as well. It was also determined that in the composition of suds dominates long chain FA (C16 - C18), and saturated FA comprises 54 % of total content of FA. The composition was also analyzed by TLC method (Fig. 22). It was determined that the main component of the suds is free FA. There are an appropriate amount of TAG and the traces of DAG and MAG as well. Thus, it is clear that lipase-catalyzed processes will include not only free FA esterification, but TAG, DAG and MAG transesterification reactions as well. Moreover, although the suds in n-hexane formed two phases, it is clear that their composition is virtually identical (Fig. 22. 1 - 2 lanes), for this reason the mixture of the suds instead of the specific phase should be used for the experiments.

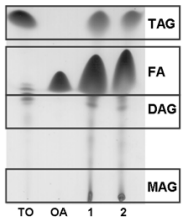


Fig. 22. Chromatographic view of the suds, extracted with n-hexane (1 – upper phase, 2 – lower phase). Controls: TO – triolein, OA – oleic acid.

The suds were dissolved in 10 different organic solvents (1:1, v/v) that can be used as substrates for lipase-catalyzed reactions. The effect of these solvents on the activity of enzymes was investigated (Fig. 23). The reaction mixture composition was as follows: the mixture of suds (with an appropriate organic solvent that acted as acyl acceptor), solvent (water, *t*-butanol or n-hexane) and enzyme – immobilized Lipex 100T or liquid Lipolase 100L. Reactions were performed at 30°C.

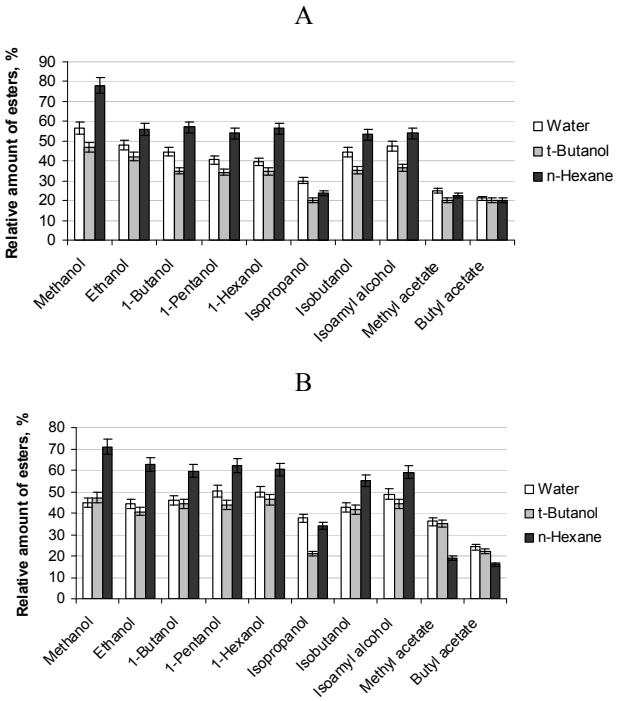


Fig. 23. The effect of various organic solvents as acyl acceptors on the productivity of Lipex 100T (A) and Lipolase 100L (B) – catalyzed transesterification of suds reaction after 5 h.

The reaction with both enzymes was more effective in n-hexane, when the mixture of suds with methanol was used. The ME yield reached 78 % and 71 % with Lipex 100T and Lipolase 100L, respectively. For this reason methanol was chosen as the best acyl acceptor for the further experiments. The suds were also dissolved in traditional solvents used for lipases-catalyzed reactions – water, *t*-butanol, toluene, n-hexane and n-heptane. The

productivity of suds transesterification with methanol reactions in these solvents catalyzed by Lipex 100T and Lipolase 100L was compared (Fig. 24).

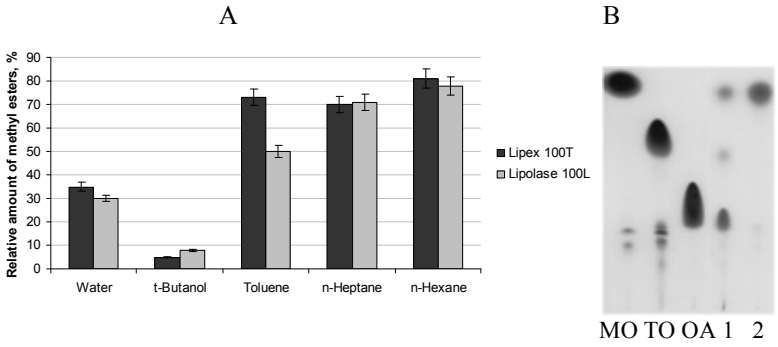


Fig. 24. The effect of solvent on productivity of Lipex 100T ir Lipolase 100L catalyzed suds methanolysis after 3 h (A). The chromatographic view of the products generated during Lipex 100T-catalyzed suds methanolysis in n-hexane after 1 h (1) and 4 h (2). Controls: MO – methyl oleate, TO – triolein, OA – oleic acid.

It was clear that the reaction was most effective n-hexane too. The ME yield obtained after 3 h reached 81 % when Lipex 100T was used. The chromatographic view of Lipex 100T catalyzed reaction is shown in Fig. 24 B, where can be seen that the reaction is completed after 4 hours (Fig. 24 B, 2 lane).

The utilization of suds as the medium for algae growth was not successful. This may be associated with the deficiency of some components such as nitrogen, phosphorus, etc. that are essential for the growth of algae.

Conclusions

1. *t*-Butanol is more sutable for hydrolysis while n-hexane is more favorable for the synthesis reactions catalyzed by lipases. The utilization of various organic solvents, new additives, drying agents and different acyl acceptors in order to enhance the productivity of lipase-catalyzed rapeseed oil methanolysis may have some negative effects, depending on the enzyme used and other parameters.

2. Oleic acid esterification with trimethylolpropane was determined to be more efficient in comparison to methyl oleate transesterification for the production of trimethylolpropane triesters. The yields obtained under mild conditions show the potential to be applied for the industrial production of biolubricants.

3. Coconut oil transesterification with β-citronellol in solvents with logP values higher or equal to +3.50 catalyzed by Lipolase 100L is an effective way to produce biotechnologically valuable terpenyl esters.

4. Depending on the structure of the carboxylic acid used for the reaction, lipases show the ability to catalyze the esterification reaction of dicarboxylic acids (glutaric and oxalic), producing corresponding diesters and other products, the number of which varies with different alcohols and enzymes used.

5. The transesterification and esterification of suds catalyzed by Lipolase 100L and Lipex 100L producing various FA esters is an effective way of utilization of these wastes.

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Reziumė

Lipazės dėl savo įvairiapusiškumo yra vieni labiausiai tyrinėjamų ir įvairių junginių sintezei naudojamų hidrolizinių fermentų, kurie, priklausomai nuo reakcijos sąlygų, gali katalizuoti net tik hidrolizę, bet ir esterių sintezę, aminolizę, epoksidaciją ir kt. Nors, palyginus su chemine katalize, fermentinei sintezei būdingi tokie pranašumai kaip švelnios reakcijų sąlygos, aukštas substratinis savitumas, šalutinių reakcijų nebuvimas, ekologiškumas, geresnės kokybės produktai ir kt., visgi ir čia susiduriama su tam tikromis problemomis: reikia didelių fermento kiekių, po keleto reakcijų ciklų nustatomas biokatalizatoriaus aktyvumo sumažėjimas, fermentas gali būti slopinimas reakcijos mišinio komponentais. Visa tai ekonomiškai nepalanku, todėl, norint sumažinti tokios gamybos kaštus, būtinas kiekvieno fermento savitumo ir aktyvumo įvertinimas skirtingų substratų, reakcijų tipo bei sąlygų atžvilgiu individualiai. Todėl darbo metu ir buvo tiriamas pasirinktų iki šiol mažai tyrinėtų lipazių savitumas bei aktyvumas, katalizuojant skirtingomis sąlygomis vykdomas reakcijas, susidarant tokiems produktams kaip biodyzelinas, biotepalai bei kiti įvairios paskirties vertingi esteriai.

Įvertinus įvairių reakcijos sąlygų įtaką biodyzelino sintezės (rapsų aliejaus (RA) transesterinimo metanoliu) produktyvumui, nustatyta, kad *tret*-butanolis yra tinkamesnis aliejų hidrolizei, o n-heksanas – transesterinimui vykdyti. Be to, vandens kiekio pokyčiai daro skirtingą įtaką reakcijų efektyvumui – kuo tirpiklis hidrofobiškesnis, tuo daugiau vandens reikia maksimaliam proceso efektyvumui pasiekti: optimali vandens koncentracija *tret*-butanolyje yra 9 %, o n-heksane - 26 %. Procesams abiejuose tirpikliuose būdinga ta pati optimali temperatūra - 40^{o}C. *tret*-Butanolyje visas aliejus suskaldomas per 1,5 val., kai molinis RA ir metanolio santykis yra 1:6 – 1:8, vanduo sudaro 9 %, o fermentinio preparato kiekis – 44 mg/ml. Didžiausia gauta esterių išeiga siekia 85 %, kai reakcija vykdyta 24 val. n-heksane, esant 26 % vandens, 66 mg/ml fermentinio preparato bei RA ir metanolio moliniam santykiui 1:12 – 1:16.

Siekiant išvengti neigiamo metanolio poveikio lipazei, neretai vietoj alkoholio naudojami jo acto rūgšties esteriai. Palyginus Lipolase 100L lipazės katalizuojamo RA transesterinimo metanoliu bei metil- ir butilacetatais, efektyvumą, paaiškėjo, kad acetatų naudojimas tirtomis sąlygomis nėra efektyvus reakcijos produktyvumo didinimo būdas. Siekiant padidinti lipazių katalizuojamų reakcijų produktyvumą, vis dažniau naudojami įvairūs priedai, tačiau darbų metu pasirinkto ektoino naudojimas tirtomis sąlygomis nepasiteisino. Kadangi ektoino kiekis (1,1 mM) buvo pasirinktas, atsižvelgus į literatūroje rastus duomenis, tikėtina, kad mūsų atveju tai nebuvo optimali jo koncentracija. Kaip žinoma, reakcijų tirpikliai turi esminės įtakos lipazių katalizuojamų reakcijų eigai, tad buvo pasirinkti nauji mažai tirti glikolio eteriai, tačiau daugeliu atveju jų naudojimas neprilygo procesams, vykstantiems tradiciniuose tirpikliuose – n-heksane bei *tret*-butanolyje, visgi reakcijos tokiuose tirpikliuose produktyvumas gali būti ženkliai padidintas, naudojant džiovinimo agentus - molekulinius sietus.

Ištyrus komercinių lipazių gebą katalizuoti sudėtingų esterių - biotepalų - sintezę, kai vienas iš substratų yra trimetilolpropanas (TMP), nustatyta, kad oleino rūgšties esterinimas TMP yra efektyvesnis trimetilolpropano trioleato (TMP-TO) gavimo būdas nei metiloleato transesterinimas. Didžiausia gauta TMP-TO esterių išeiga siekė 62 %, o bendra TMP tri-, di- ir monoesterių išeiga sudarė 83 bei 85 % Lipoprime 50 T lipazės katalizuojamos

reakcijos metu po 72 val. bei Lipozyme RM IM – po 96 val., atitinkamai. Nors Lipozyme RM IM katalizuojama reakcija yra ilgiau trunkantis procesas, tačiau tik jis potencialiai gali būti taikomas didelio masto TMP-TO gamybai, nes vyksta bevandenėje aplinkoje, o fermento kiekis sudaro tik 15 % (w/w), kai tuo tarpu Lipoprime 50T - net 40 % (w/w).

Buvo siekiama atrinkti ir naujus lipazių katalizuojamoms vertingų esterių sintezės reakcijoms tinkamus substratus bei įvertinti įvairių sąlygų įtaką susidarančių produktų išeigoms. Atlikus naujų substratų (acilakceptorių), tinkamų Lipolase 100L ir Lipex 100L lipazių katalizuojamam RA transesterinimui, atranką, nustatyta, kad tirtomis sąlygomis gana efektyviai vyksta RA transesterinimo dviem terpenoliais - β-citroneloliu bei geranioliu – reakcijos. Palyginus minėtų lipazių katalizuojamų vienuolikos skirtingų aliejų transesterinimo β-citroneloliu reakcijų *tret*-butanolyje bei n-heksane efektyvumą, paaiškėjo, kad daugeliu atvejų sintezė efektyvesnė n-heksane, o iš visų tirtų aliejų produktyviausiai transesterinamas kokosų aliejus (KA). Kadangi didžiąją KA sudėtyje esančių riebalų rūgščių (RR) dalį sudaro sočiosios RR, iš kurių apie 46 % - lauro rūgštis (C12:0), galima daryti išvadą, kad tirti fermentai yra savitesni sočioms trumpesnės anglies grandinės RR. Atlikus aktyviausių tiriamo KA transesterinimo β-citroneloliu atžvilgiu fermentų atranką, nustatyta, kad reakcija efektyviausiai katalizuojama Lipolase 100L, Lipozyme TL100L bei Resinase A2X - po 24 val. susidariusių esterių kiekis viršija 70 %. Įvertinus 20 pasirinktų organinių tirpiklių įtaką Lipolase 100L katalizuojamo KA transesterinimo β-citroneloliu efektyvumui, paaiškėjo, kad tirtomis sąlygomis reakcija produktyviausiai vyko hidrofobiniuose tirpikliuose, kurių logP vertė didesnė arba lygi 3,50 - n-oktane, izooktane, n-heptane, cikloheksane ir n-heksane – čia susidariusių esterių išeigos siekė 72 %.

Ištyrus pasirinktų komercinių lipolizinių fermentinių preparatų gebą katalizuoti įvairių vieną ar daugiau hidroksi ir/ar karboksigrupių turinčių organinių rūgščių esterinimą β-citroneloliu bei oleilo alkoholiu, nustatyta, kad tirtomis sąlygomis iš visų pasirinktų rūgščių (oksalo, glutaro, ftalo, galo, citrinų) reakcijos vyksta tik su glutaro ir oksalo rūgštimis. VU Chemijos fakultete ^{1}H BMR spektroskopijos ir GC-MS analizės metodais patvirtinta, kad Novozym 435 lipazės katalizuojamo glutaro rūgšties esterinimo β-citroneloliu metu susidaro glutaro rūgšties β-citronelolio diesteris. Įdomu tai, kad oksalo rūgšties esterinimo β-citroneloliu ir oleilo alkoholiu metu gaunamas skirtingas produktų skaičius, kuris kinta ir naudojant skirtingus komercinius lipolizinius fermentus.

Buvo įvertintos ir muilo gamybos riebalinių atliekų – pamuilių – utilizavimo galimybės, jas fermentiškai esterinant/transesterinant. Tyrimų metu nustatyta, kad didžiąją šiose atliekose esančių riebalinių komponentų dalį sudaro laisvos RR: dominuoja ilgos grandinės RR (C16 - C18), o sočiosios sudaro 54 % viso pamuilėse esančių RR kiekio; aptinkama ir tri-, di- ir monoacilglicerolių. Nustatyta, kad fermentinis pamuilių esterinimas/transesterinimas, susidarant įvairiems RR esteriams, yra efektyvus pamuilių utilizavimo būdas. Lipex 100T katalizuojamo n-heksanu ekstrahuotų pamuilių esterinimo/transesterinimo metanoliu, esant 30^{o}C temperatūrai, metu po 4 val. susidariusių esterių išeiga siekia praktiškai 100 %. Pamuilių, kaip dumblių auginimo terpės, panaudojimas pasirodė esąs neveiksmingas. Tai gali būti susiję su kai kurių pagrindines gyvybines dumblių funkcijas užtikrinančių makrokomponentų (azoto, fosforo junginių bei kitų mineralinių medžiagų) trūkumu.

CURRICULUM VITAE

Name Vita Kiriliauskaitė

Date of birth July 1, 1986

Address Department of Biochemistry and Molecular Biology
Vilnius University
M. K. Čiurlionio 21/27, LT-03101
Vilnius
Lithuania

E-mail vita.kiriliauskaite@gmail.com
vita.kiriliauskaite@gf.vu.lt
Phone +370 5 239 8244

Education

2010 - 2014	PhD studies, Biochemistry Vilnius University, Lithuania
2008 - 2010	M. Sc., Biochemistry Vilnius University, Lithuania
2004 - 2008	B. Sc., Biochemistry Vilnius University, Lithuania

Professional Experience

2011 - present	Junior Researcher Vilnius University, Lithuania
2009 - 2010	Senior Specialist Vilnius University, Lithuania
2008 - 2009	Specialist Vilnius University, Lithuania

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