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Enzymatic phenethyl octanoate synthesis: lipase selection and reaction optimization by response surface methodology

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Phenethyl octanoate (PEO) is a natural flavour compound found in rum, spirits and wine. Six commercial and non-commercial lipase preparations were screened for 2-phenethyl octanoate synthesis activity, the most active enzymes were Lipozyme® RM IM and Palatase® 20000 L (immobilized and soluble *Rhizomucor miehei* lipases). Three substrates acyl-donors (octanoic acid, glyceryl trioctanoate and coconut oil) and two solvents (hexane and *tert*-butanol) were compared in the synthesis reactions. Higher conversions and reaction rates were observed in hexane using immobilized enzyme. The optimal reaction conditions determined by means of Response Surface Methodology (RSM) were as follows: reaction time 120 minutes, temperature of 30 °C, 0.8 M and 0.267 M concentrations of 2-phenylethanol and glyceryl trioctanoate, respectively, 7% concentration of enzyme Lipozyme® RM IM, resulting in 80% reaction conversion. The phenylethyl ester synthesis reaction was transferred to a solvent-free system using a natural substrate coconut oil.

Key words: phenethyl octanoate, flavor esters, lipase, *Rhizomucor miehei*, response surface methodology (RSM)

INTRODUCTION

A large variety of compounds determine the flavour of food products, including alcohols, aldehydes, esters, dicarbonyls, short to medium-chain free fatty acids, methyl ketones, lactones, phenolic compounds and sulphur compounds. Since early times humankind has extracted flavour compounds from natural sources. Nowadays flavour esters are widely used in food, fragrance and cosmetic industry. Flavours represent over a quarter of the world market for food additives, most of the flavouring compounds are produced via chemical synthesis or by extraction from natural materials. Even though flavour compounds often occur naturally in plant and animal tissues, the extraction process is costly due to low quantities in the tissues [1].

Flavour esters are usually produced by conventional chemical synthesis which seldom is environmental-friendly, because polluting and corrosive inorganic catalysts are commonly used. In addition, it lacks specificity and selectivity, which may cause the formation of undesirable racemic mixtures or other by-products, thus reducing process efficiency and increasing downstream costs [1, 2].

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Consumer preferences to the products labelled "natural" and "ecological" increased the demand for naturally produced flavour compounds. Scientific interest was drawn to the biotechnological, especially, enzymatic synthesis of flavour compounds [3], as mild reaction conditions with high specificity minimize side-reactions and allow easier and cheaper separation of reaction products. Enzymatic synthesis is an attractive alternative especially when food, flavour and fragrance compounds are in scope, because while avoiding harsh reaction conditions, high yields are achieved and natural compounds are synthesized from natural and renewable sources [4]. Perfumery and flavour grade esters need to be free of inorganic impurities; reduced reaction temperature and increased yield reduce the process costs, therefore enzymatic catalysis is an alternative, especially when food-grade enzymes are employed.

Phenethyl octanoate (synonyms 2-phenylethyl octanoate, phenethyl caprylate) is a naturally occurring flavour compound in wines and spirits [5], okra (*Abelmoschus esculentus*) seed coat [6]. 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. Some species of ants like *Camponotus arminius* and *Crematogaster clarithorax* use it in their chemical communication system [7].

Attempts have been made to synthesize phenethyl esters enzymatically from natural sources of octanoic acid, especially coconut oil [8, 9]. Coconut fat is rich in short-chain fatty acid of C8 (octanoic acid, 7.5% w/w) and coconut cream is produced from coconut milk after centrifugation and contains about 25% (w/v) of fat. Coconut milk is cheap and readily available in many countries of the world. Both coconut fat and coconut cream may be attractive substrates for enzymatic production of natural esters [10, 11].

In order to obtain high yields of a desired product, enzyme and reaction conditions have to be selected carefully.

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) and various acyl-donor substrates: octanoic acid (caprylic acid), glyceryl trioctanoate (reaction scheme in Fig. 1) and coconut oil (a natural source of octanoic acid).

EXPERIMENTAL

Materials

Lipases Palatase® 20000 L (further in the text Palatase®), Lipozyme® RM IM, Lipoclean®, Lipopan® F BG were kindly donated by JSC Biopolis, Novozymes representative in Lithuania. Lipase from *Enterobacter aerogenes*-13 (190 U/ml) was provided by JSC BIOCENTRAS, Vilnius, Lithuania. Lipase from *Pseudomonas mendocina* 3121-1 (70 U/ml) was provided by the Institute of Biochemistry, Vilnius, Lithuania. The reagents of analytical grade or >=99% were purchased from Sigma-Aldrich (St. Louis, MO, USA): 2-phenylethanol (2-PE), phenethyl octanoate (PEO), octanoic acid (OA), glyceryl trioctanoate (GTO), coconut oil, hexane, *tert*-butanol, diethyl ether, petroleum ether, acetic acid. Pre-coated thin-layer chromatographic (TLC) plates DC-Fertigplatten SIL G-25 UV_{254} were purchased from Macherey-Nagel (Düren, Germany).

PEO synthesis

The ester synthesis was performed in 2 ml capped test tubes at least in duplicate, response surface design (RSM) experiments were performed according to Table 1, in equimolar ratios of 2-PE and OA (taking into consideration that GTO stoichiometrically contains 3 acyl-moieties). In a standard procedure the initial concentrations of substrates were 1 M, reaction was started by adding an appropriate mass or volume of lipase to the preheated substrate solution in a solvent (hexane or *tert*-butanol). Enzyme concentration was 5% v/v or m/v for screening reactions and 4.2% for other reactions, if not stated otherwise. The total volume of the reaction mixture was 1 ml.

Samples were incubated in a Biosan Thermoshaker (TS-100) at 30–60 °C, 1 400 rpm, along with controls. For further analysis aliquots of 20 µl, taken from the samples, were mixed with equal volumes of cold diethyl ether and frozen at –20 °C.

Analysis

Quantitative thin layer chromatography (TLC). Analysis of the reaction products was carried out on TLC plates (5 × 10 cm and 10 × 10 cm) pre-coated with 0.25 mm Silica Gel 60 UV_{254}

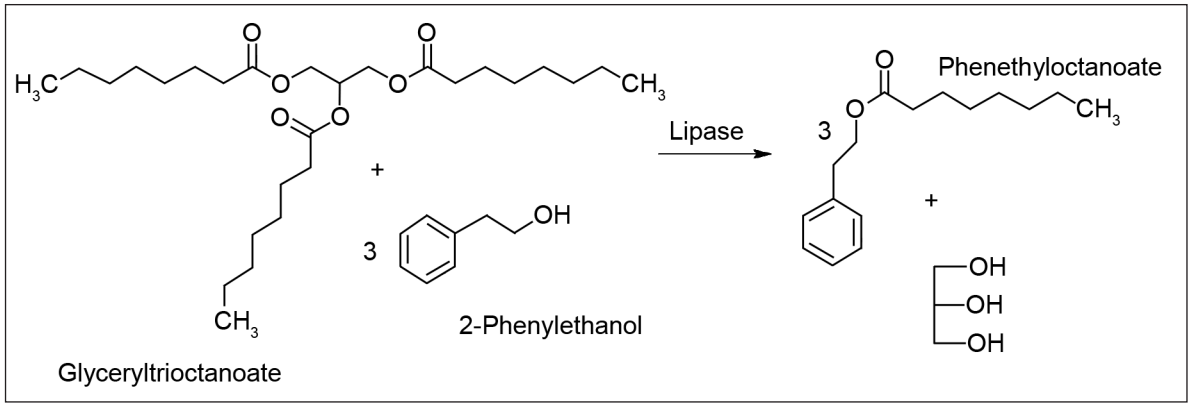

Fig. 1. Enzymatic phenethyloctanoate synthesis shceme

Table 1. **Central composite design parameter values used for the phenethyl octanoate synthesis reaction optimization by RSM**

Run	Factor A: Enzyme concentration, %	Factor B: Substrate concentration, mM	Factor C, Temperature, °C	Factor D, Time, min	Response: Phenethyl-octanoate yield, %
1.	7	0.8	40	60	64.9
2.	7	0.8	40	120	72.6
3.	4.5	1.4	35	90	59.1
4.	9.5	1.4	35	90	65.3
5.	4.5	1.4	35	90	55.4
6.	0.1	1.4	35	90	21.5
7.	2	2.0	40	120	42.4
8.	2	2.0	30	60	36.2
9.	4.5	2.6	35	90	49.6
10.	7	2.0	30	120	53.8
11.	4.5	1.4	25	90	49.3
12.	2	0.8	30	120	62.4
13.	4.5	1.4	35	30	50.0
14.	4.5	1.4	35	90	57.4
15.	4.5	1.4	35	90	53.1
16.	2	2.0	40	60	32.0
17.	7	0.8	30	60	77.1
18.	4.5	0.2	35	90	79.3
19.	4.5	1.4	35	90	56.8
20.	2	0.8	30	60	52.1
21.	7	2.0	40	60	48.1
22.	7	0.8	30	120	82.2
23.	2	0.8	40	60	47.7
24.	7	2.0	30	60	52.8
25.	2	2.0	30	120	44.3
26.	4.5	1.4	45	90	56.0
27.	2	0.8	40	120	52.5
28.	7	2.0	40	120	54.3
29.	4.5	1.4	35	90	55.6
30.	4.5	1.4	35	150	65.5

(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 5 µl 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 °C): diethyl ether : acetic acid (85:15:2, v/v) (a modified method of Sinkuniene et al. [12]). 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), all spots (including OA and GTO) were visualised after spraying the TLC plates with 0.2% 2,7-dichloroflouresceine solution in 2-propanol and drying on 30 °C surface for one hour. Pure 2-PE, OA, GTO and PEO solutions in hexane were used as standards.

Quantitative analysis of reaction products separated by TLC was performed using the principles of densitometry by the 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.

Gas chromatography-mass spectrometry by Perkin-Elmer GC-MS was used to identify the reaction product – phenethyl octanoate (NIST database was used). Hydrogen was used as a carrier gas, detector temperature was 350 °C, and starting temperature of a column was 50 °C with a gradient of 10 °C/min increased to 310 °C.

Response surface methodology

Experimental design

In order to determine the optimal reaction conditions a five-level-four-factor central composite design (CCD) was employed, resulting in 30 experimental runs. Each numeric factor is varied over 5 levels: plus and minus alpha (axial points, alpha = 1.76, a total of eight points), plus and minus 1 (factorial points, a total of 16 points) and the centre point (centre points had six replicates).

The variables selected for the GTO transesterification with 2-phenylethanol were four numeric factors: enzyme concentration (2–7%), substrate concentration (0.8–2 M), time (60–120 min), temperature (30–40 °C). The experiment design in actual values is shown in Table 2.

The experimental design, data analysis and regression model building were performed using the Design Expert software (version 8.0.1.0, Stat-Ease Inc., Minneapolis, MN).

Data analysis

The experimental data were analyzed using the 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 transesterification 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. The 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

Enzyme screening and selection

Possibility to synthesize a desired product in a good yield depends not only on a successful selection of an enzyme catalyst but also on a careful choice of reaction conditions. All lipases naturally catalyze hydrolysis of triglyceride ester bonds, but the specificity and selectivity for certain substrates vary a lot [13]. Usually the reason for different enzyme selectivity lies behind the structure of an active site, but knowledge of the active site structure does not yet permit to forecast the enzymes specificity or selectivity. This is also true for lipases [14]. Since different lipases from varied sources have distinct substrate selectivity and specificity, it is valuable to know their ability to produce different esters.

It was previously shown by Tan et al. that commercial preparations of fungal lipases from *Rhizomucor miehei*, *Candida rugosa* and *Aspergillus niger* are capable to catalyze PEO synthesis from coconut milk (containing OA in triglyceride composition) and 2-PE [8], but additional data concerning the phenethyl octanoate yield or optimized synthesis conditions was necessary for further implementation. Li et al. synthesized phenethyl-esters from butter oil and have shown that phenethyl octanoate synthesis can be effectively catalyzed by Lipozyme TL IM [15]. Here we attempted to define a suitable enzyme preparation and optimal reaction conditions for 2-PEO synthesis.

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

Immobilized (Lipozyme® RM IM) and soluble (Palatase®) preparations of *Rhizomucor miehei* lipase (produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism) were selected to determine the influence of the catalyst physical state [16]. Other two lipase preparations of Novozymes were selected with expectation to have a wide range of substrate specificity, as suggested by their standard application: Lipoclean® is used for laundry detergents while Lipopan® for baking industry [16]. Lipolase® L EX was chosen because of an outstanding esterification and transesterification activities in biodiesel synthesis reactions, as shown by our group (not published data). *Enterobacter aerogenes* lipase was selected for screening, because it has the highest activity in hydrolysis of medium chain-length fatty acid esters, in particular octanoic acid nitrophenyl ester [17] and *Pseudomonas mendocina* lipase was chosen because of its property to have a higher relative activity for natural substrate coconut oil than longer-fatty acids containing triolein or olive oil [18].

From the screening results, 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. There was no esterification activity in cases of Lipopan F BG or lipases from *E. aero-*

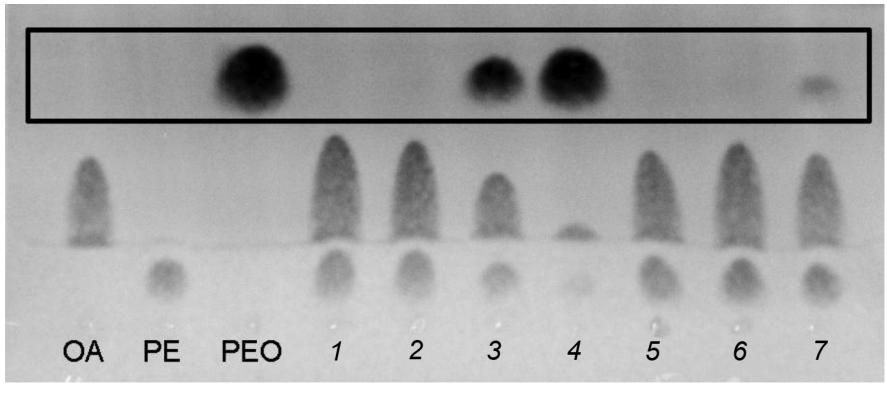

Fig. 2. Lipase screening for 2-phenethyl octanoate (PEO) synthesis reaction: esterification of octanoic acid (OA) with 2-phenylethanol (2-PE). Reaction time: 2 hours, 30 °C. Lanes: standards of OA, PE, PEO; reaction catalyzed by Lipopan® F BG (*1*), Lipoclean® (*2*), Palatase® (*3*), Lipozyme® RM IM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

Table 2. **Lipase screening results for 2-phenethyl octanoate synthesis reaction (OA + PEO). Only active lipases are presented**

Enzyme	Ester yield* in screening reactions, %	
	2 h	24 h
Palatase®	33.5	67.3
Lipozyme® RM IM	63.8	70.8
Lipolase® 100 L EX	4.1	62.9

* Standard deviation is 1.51.

genes and *Ps. mendocina*. The screening reaction yields after 2 and 24 hours are represented in Table 2.

In two hours high conversion was obtained using both soluble and immobilized *Rhizomucor miehei* lipase, therefore the preparations were selected for further experiments to determine the optimal reaction conditions.

Influence of solvent on PEO yield

Several conventional organic solvents were chosen, because the substrates and products of PEO synthesis reaction are scarcely soluble or insoluble in water. In addition, aqueous media could result in an increased hydrolysis of the reaction product. Lipase activity and specificity usually depends on physicochemical properties of solvents, especially hydrophobicity [19, 20]. Therefore two solvents with different polarities were chosen: n-hexane being a non-polar solvent ant *tert*-butanol a polar solvent with 1-octanol / water partition coefficient Log P values of 4.00 and 0.35, respectively [21]. It was previously shown that esterification of undecanoic acid with glycerol catalyzed by *Rhizomucor miehei* lipase (Lipozyme® IM20) resulted in the greatest ester yields in solvents of the highest log P (4.0–4.5) [22].

We have chosen three different substrates for PEO synthesis: OA, GTO and coconut oil. OA serves as a substrate for esterification reaction and the latter two are substrates for transesterification reactions. Coconut oil is a natural triglyceride substrate with a mixture of fatty acids of various lengths and saturations [23], therefore GTO was chosen as a model substrate to demonstrate that the targeted OA is incorporated into PEO. Triglycerides containing octanoic acid are found not only in coconut oil, but also in dairy fat (butter), which can be used as a complex substrate [8].

Both enzyme preparations were more active in hexane compared to *tert*-butanol (Fig. 3). Although higher ester yields in 6 hours are obtained in hexane (63% Lipozyme RM IM), initial reaction rates depend more on the type of the catalyst used, and are higher for immobilized enzyme preparation both in hexane and *tert*-butanol. The reaction with the immobilized enzyme reaches plateau in 1–3 hours, whereas reactions catalyzed by Palatase® do not seem to reach equilibrium after 6 hours in both solvents.

The same tendencies are observed for GTO substrate: a lower reaction yield in 24 hours is obtained in *tert*-butanol (Fig. 4). Ester yield is especially low when using a soluble enzyme (Palatase®) in *tert*-butanol, in this case the conversion is only 4.3% after 24 hours. The highest yield is observed with Lipozyme® RM IM in hexane, and reaches about 65% in six hours and does not increase further. If Lipozyme® RM IM in *tert*-butanol is used, the reaction also reaches equilibrium after one hour, but the conversion is only 40%.

If coconut oil substrate is used, the trend of much faster reaction with Lipozyme® RM IM and higher yields in hexane remains the same. The main difference in using a coconut oil substrate for transesterification with 2-PE is that the concentration increases even after 6 hours of reaction in all cases and reaches over 80% in the case of Lipozyme® RM IM in hexane (Fig. 5). Different curve pattern could be observed due to the different enzyme specificity to different fatty acids present in coconut oil [23]. *Rhizomuor miehei* lipase similarly to other lipases has different selectivity for various chain-length fatty

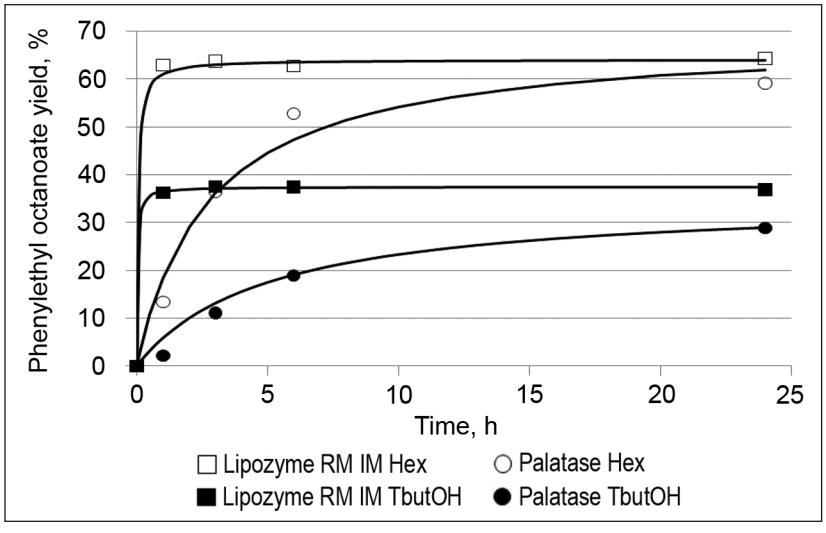

Fig. 3. Esterification of octanoic acid with 2-phenylethanol, catalyzed by Palatase® and Lipozyme® RM IM enzymes at 30 °C, in hexane and *tert*-butanol

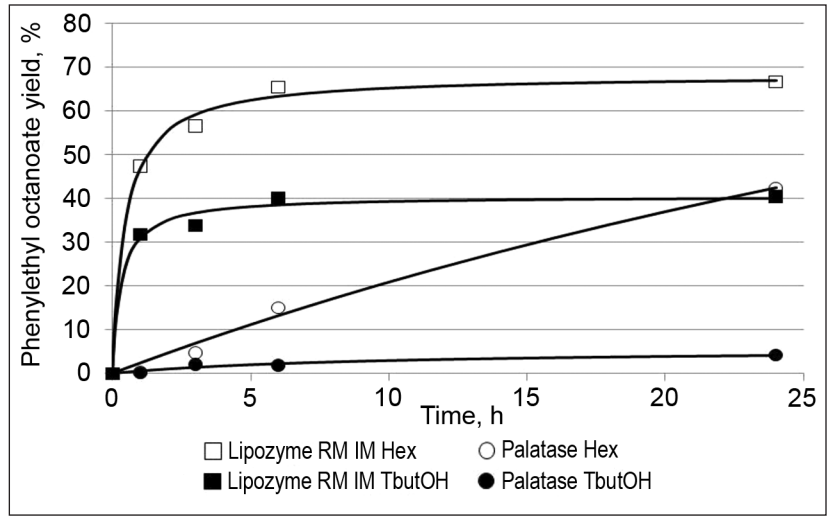

Fig. 4. Transesterification of gliceryltrioctanoate with 2-phenylethanol, catalyzed by Palatase® and Lipozyme® RM IM enzymes at 30 °C, in hexane and *tert*-butanol

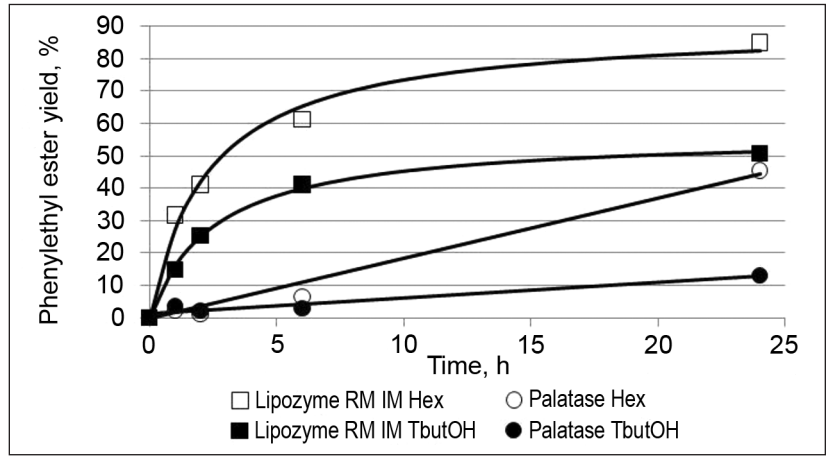

Fig. 5. Transtesterification of coconut oil with PE, catalyzed by Palatase® and Lipozyme® RM IM enzymes at 30 °C, in hexane and *tert*-butanol

acid glyceryl esters present in coconut oil (most of them have a longer aliphatic chain compared to octanoic acid). If the enzyme had a higher selectivity towards shorter (including octanoic) acid esters, the synthesis of phenethyl octanoate from coconut oil in shorter reaction times would be preferred due to higher proportion of reacted favourable fatty acid. More experimental data is needed to make conclusions.

Optimal reaction conditions determined by response surface methodology

Response surface methodology (RSM) consists of a group of mathematical and statistical techniques used to develop an adequate functional relationship between a response of interest and a number of associated controllable variables. Determining the relationships between several variables using the RSM means varying several parameters at a time, according to the statistically built-up design, which can be represented by a matrix [24]. In contrast to the classical technique which varies only one parameter at a time, is time consuming and cannot predict the parameter interactions, RSM is a faster and less expensive technique which provides sufficient information for statistically acceptable results [25].

Simple fruit flavour ester synthesis (*n*-butyl acetate, *n*-propyl acetate, isoamyl butyrate, ethyl butyrate and others) is commonly optimized by RSM [26–30], in most cases esters are synthesized via direct esterification reaction. There are some examples of transesterification reactions involving 2-PE. Rose aromatic ester (2-phenylethyl acetate) was synthesized 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 (Novozyme® 435) for 50 mM substrate concentrations in hexane [31]. Because of the importance to have a method for "natural" ester synthesis, for optimization reaction we have chosen a triglyceride GTO substrate which resembles the reaction where natural fats are used for the synthesis.

According to the data presented in 3.1, immobilized lipase Lipozyme® RM IM and hexane solvent were chosen. The influence of four reaction setup factors (enzyme and substrate concentrations, temperature and time) on PEO yield was determined.

The experiment design and response in actual values is shown in Table 2. The regression calculations were done by the Design Expert program to fit the polynomial models to the selected response. A 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. The ANOVA is presented in Table 3. The model equation in actual factors is presented in Table 4. The model is highly significant with a p-value of <0.0001 and the adjusted multiple correlation coefficient $R^2 = 0.9167$.

The mutual effect of parameters can be better understood by examining the response surface charts. In a three-dimensional chart two variables can be explored, the others have to be set to constant values. In Fig. 6 the influence of enzyme and substrate concentrations to the reaction yield is plotted:

Table 3. **ANOVA for the response surface quadratic model**

Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Model	4 718.05	8	589.76	40.89	<0.0001	Significant
A-Enzyme concentration	2 164.95	1	2 164.95	150.09	<0.0001	
B-Substrate concentration	1 788.02	1	1 788.02	123.96	<0.0001	
C-Temperature	45.15	1	45.15	3.13	0.0914	
D-Time	297.53	1	297.53	20.63	0.0002	
AB	48.51	1	48.51	3.36	0.0809	
BC	40.25	1	40.25	2.79	0.1097	
A^2	292.03	1	292.03	20.25	0.0002	
B^2	144.09	1	144.09	9.99	0.0047	
Residual	302.90	21	14.42			
Lack of fit	281.89	16	17.62	4.19	0.0604	Not significant
Pure error	21.01	5	4.20			
Cor total	5 020.95	29				

Table 4. **Model coefficients for equation in terms of actual factors**

Term	Model coefficient
Intercept	+76.85148
Enzyme concentration	+10.60914
Substrate concentration	–45.14129
Temperature	–1.01448
Time	+0.11737
Enzyme conc.·Substrate conc.	–1.16082
Substrate conc.·Temperature	+0.52868
Enzyme $conc.^2$	–0.56571
Substrate $conc.^2$	+6.24123

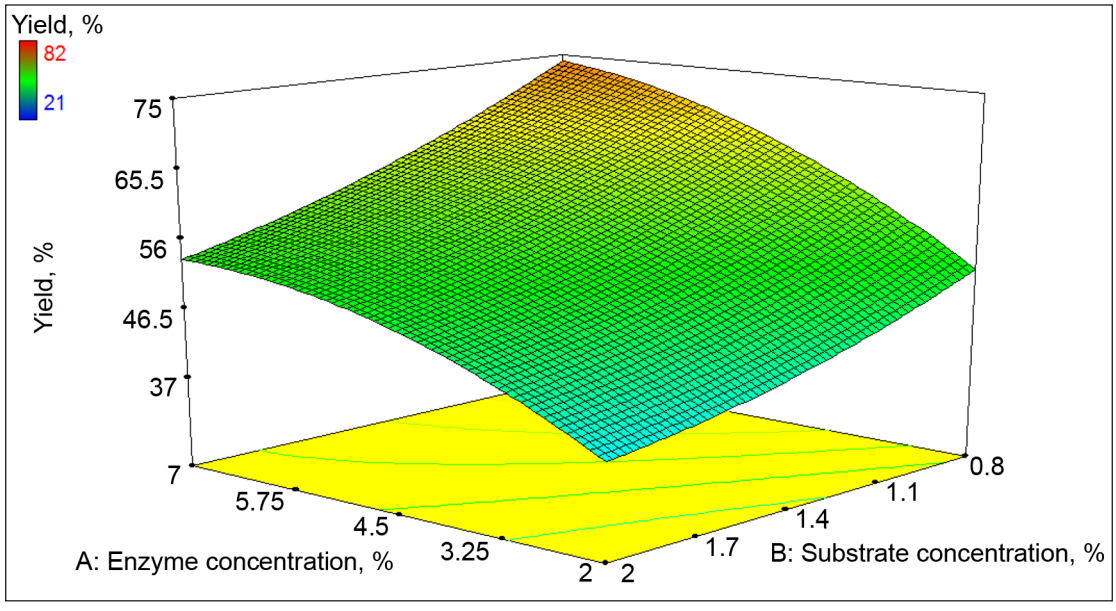

Fig. 6. Response surface plot showing the mutual effect of substrate and enzyme concentrations on the reaction yield (conversion, %), temperature and time are set to mean values (i. e. 35 °C and 90 min)

the higher the enzyme concentration and the lower the substrate concentration, the higher is the reaction yield. It is also observed in Table 4 that the influence of enzyme concentration is positive and the influence of substrate concentration is negative, that could be due to the enzyme saturation by substrate. In the temperature of 35 °C and 90 minutes of the reaction time the yields vary from 37% (enzyme concentration 2%, substrate concentration 2 M) to 75% (enzyme concentration 7%, substrate concentration 0.8 M).

The effect of the reaction temperature is also negative, which means catalysis works well in lower temperatures. It is not surprising that the effect of the reaction time is positive, but as it can be observed from Fig. 7, even when approaching 120 min, the yield is still increasing. Therefore it should be considered that longer reaction times, if they are acceptable, might increase the PEO 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 as follows: 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 performed in the given conditions and the obtained yield was 80.0%. The results correlate well with the predicted data.

Jungbae et al. have synthesized various flavour esters enzymatically, using enzyme mixtures. The PEO yield was 64% after 20 hours using 10 mM octanoic acid, 20 mM phenylethanol and 2 mg/ml of each enzyme (*Penicillium roqueforti*, *Candida rugosa* and *Aspergillus niger*) in hexane [32]. In our case the conversion was higher after ten-fold shorter reaction time. Similarly longer reaction time was also applied in the study of Li et al. which have synthesized phenethyl octanoate from 2-phenylethanol (125 mM) and octanoic acid (150 mM) hexane and achieved 84% yield in 24 hours catalyzed by 5% w/w (reactants) of Lipozyme TL IM [15]. Reaction conditions optimized in our study offer high productivity in a shorter reaction time, 80.0% conversion can be obtained in as short as two hours.

Transesterification of coconut oil with 2-phenyl ethanol

Rhizomucor miehei lipase was tested to carry out the natural substrate – coconut oil – transesterification reaction in solvent-free conditions. Coconut oil is one of the most popular natural sources rich in octanoic acid. The possibility to avoid conventional solvent usage (substrates act as solvents themselves) provides easier purification because fewer components are present in the final reaction mixture. The process does not require solvents therefore it is more favourable for the environmental-friendly industrial application. The reaction product is a mixture of different acid chain length esters and can be used not only after further purification but also directly in food products, such as cheese [15].

A liquid form of lipase Palatase® was tested out for this reaction because a better distribution of enzyme was expected in a relatively viscous reaction mixture.

The reaction was conducted by a step-wise addition of alcohol (three times 24 hours apart in equal portions with the final concentration of two alcohol molecules per one acyl moiety). The 2-phenylethyl esters yield reached 37%

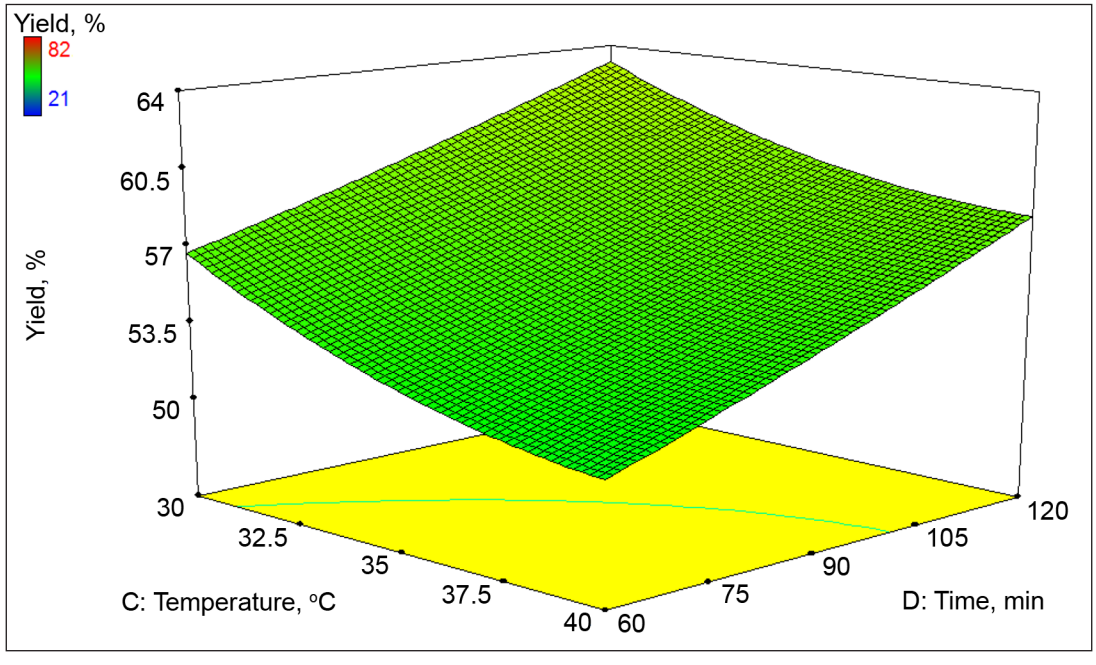

Fig. 7. Response surface plot showing the mutual effect of reaction temperature and time on the reaction yield (conversion, %), enzyme and substrate concentrations are set to mean values (i. e. 4.52% and 1.4 M, respectively)

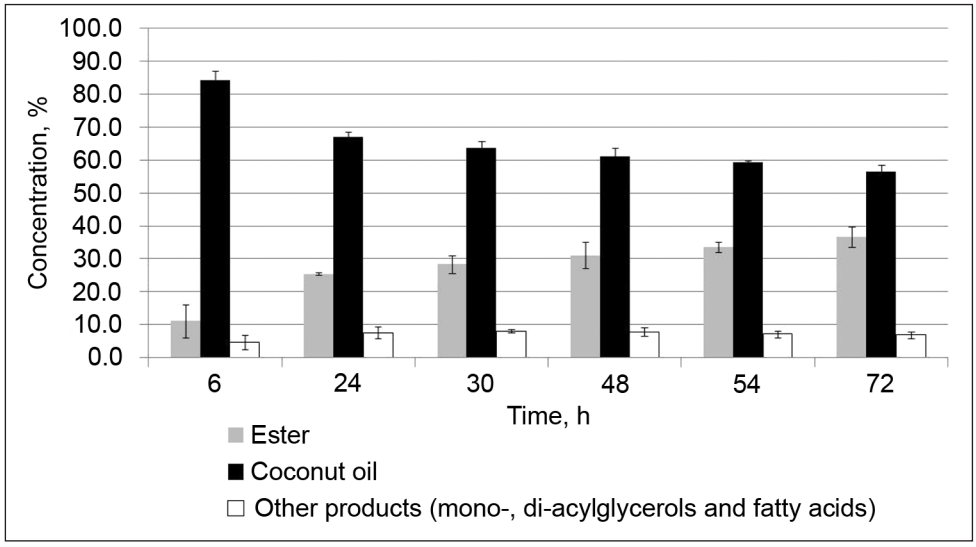

Fig. 8. Transesterification of coconut oil by Palatase® (4.2%) in a solvent-free medium at 30 °C

and 54% of oil was transesterified in 72 hours (Fig. 8). The reaction conversion was not as high as for trioctanoin substrate in a solvent. One probable cause for a lower yield is a slower mass-transfer due to higher viscosity, other causes are enzyme stability and phase limitations. Li et al. have used enzymatic synthesis to produce a mixture of 2-phenethyl esters in solvent-free conditions using butter-oil as a substrate. Under the optimised conditions of the immobilized enzyme Lipozyme TL IM-catalysed reaction, the total yield of C4–18 2-phenethyl esters was about 817 mg per gram of butter oil. The optimal reaction conditions led to an 80.0% conversion of 2-phenethyl alcohol [15]. Herein with high probability after selection of proper reaction conditions the immobilized *Rhizomucor miehei* lipase Lipozyme RM IM could also successfully be used for coconut oil transesterification in a solvent-free medium.

CONCLUSIONS

Increasing attention for the synthesis of "natural" compounds used in flavour, fragrance and food industry makes biotechnological processes more attractive, especially when food-grade enzymes can be used. Phenethyl octanoate is a fragrance compound that is found and can be used in production of alcoholic beverages and fragrance applications. The synthesis of phenethyl octanoate has not yet been thoroughly researched and optimized; therefore selection of applicable enzymes and reaction conditions was done.

PEO can successfully be synthesized enzymatically using an immobilized lipase from *Rhizomucor miehei* (Novozymes® Lipozyme® RM IM) in a hexane solvent. Soluble lipase (Palatase®) is also active. PEO can be synthesized by direct esterification of oleic acid or by transesterification of gliceryltrioctanoate, which was further optimized using response surface methodology. Reaction yield is higher in lower temperatures (30 °C), 80% yields can be expected in as short as two hours when 7% of lipase preparation is used. Coconut oil, containing octanoic acid in the triglyceride composition, can be transesterified in a solvent free medium, and even though the conversion is lower compared to the solvent-system, there is a high potential to increase it by choosing appropriate reaction conditions.

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FERMENTINĖ FENETILOKTANOATO SINTEZĖ: LIPAZIŲ ATRANKA IR REAKCIJŲ OPTIMIZAVIMAS ATSAKO PAVIRŠIAUS METODOLOGIJA

S a n t r a u k a

Nuolat didėjantis susidomėjimas natūraliais junginiais, naudojamais kvepalų ir maisto pramonėje, lemia vis didėjantį biotechnologinio gamybos proceso populiarumą. Fenetiloktanoatas – kvapusis esteris, aptinkamas spirituotame vyne ir kai kuriuose augaluose, gali būti naudojamas alkoholinių gėrimų ir kvepalų gamyboje. Fenetiloktanoato fermentinė sintezė iki šiol nebuvo detaliai ištirta ir optimizuota, todėl atrinkome tinkamus fermentus bei optimalias reakcijos sąlygas. Iš tirtųjų fenetiloktanoato sintezei atrinkti fermentiniai preparatai Palatase® ir Lipozyme® RM IM bei tirpiklis heksanas. Tirta galimybė sintetinti fenetiloktanoatą iš skirtingų substratų: oktano (kaprilo) rūgšties (esterinimas), trikaprilino (TK) bei kokosų aliejaus (peresterinimas). Po 6 reakcijos valandų pasiektas 75 % konversijos laipsnis naudojant Lipozyme RM IM ir TK substratą heksane; visais atvejais reakcija geriau vyksta heksane. Imobilizuotas preparatas Lipozyme® RM IM peresterinimo reakcijų metu yra žymiai efektyvesnis už Palatase®, o šis pakankamai aktyvus (konversijos laipsnis didesnis nei 60 %) tik vykdant esterinimo reakciją heksane. Taikant atsako paviršiaus metodologiją nustatyta trikaprilino peresterinimo 2-feniletanoliu konversijos priklausomybė nuo reakcijos trukmės, temperatūros, alkoholio bei fermento koncentracijos. Optimaliomis sąlygomis (trikaprilino koncentracija – 0,8 M, temperatūra – 30 °C, reakcijos trukmė – 2 val., fermento koncentracija – 7 %) gauta fenetiloktanoato išeiga – 80 %. Nauja ir originalu, kad, palyginti su literatūroje aptinkamais duomenimis, tokia didelė išeiga pasiekiama per trumpą reakcijos laiką.