www.shd.org.rs

Journal of the Serbian Chemical Society

JSCS@tmf.bg.ac.yu • www.shd.org.rs/JSCS

J. Serb. Chem. Soc. *73* (12) 1139–1151 (2008)
JSCS–3793

UDC 547.521–326:542.913+577.15
Original scientific paper

Lipase catalyzed synthesis of flavor esters in non-aqueous media: Optimization of the yield of pentyl 2-methylpropanoate by statistical analysis

ZORICA KNEŽEVIĆ-JUGOVIĆ[1]*#, DEJAN BEZBRADICA[1#], ŽIVANA JAKOVLJEVIĆ[2], SUZANA BRANKOVIĆ-DIMITRIJEVIĆ[1#] and DUŠAN MIJIN[1#]

[1]*Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11120 Belgrade and* [2]*Faculty of Mechanical Engineering, University of Belgrade, Kraljice Marije 16, 11120 Belgrade, Serbia*

(Received 19 March, revised 3 June 2008)

Abstract: In this study, the synthesis of pentyl 2-methylpropanoate employing a commercial lipase from *Candida rugosa* was investigated, the emphasis being placed on analyzing the effects of various process conditions on the yield of ester. The response surface methodology (RSM) and five-level-five-factor central composite rotatable design (CCRD) were used to evaluate the effects of variables, namely the initial water content, 0.0–2.0 % (w/v), the reaction temperature, 35–75 °C, the enzyme concentration, 1.0–5.0 g dm^{-3}, the acid/alcohol mole ratio, 1:2–5:2, and the reaction time, 4–48 h, on the yield (%) of ester. The production of pentyl 2-methylpropanoate was optimized and an ester yield response equation was obtained, enabling the prediction of ester yields from known values of the five main factors. It seems that the enzyme concentration, reaction time and acid/alcohol mole ratio predominantly determine the conversion process, while the amount of added water amount had no significant influence on the ester yield. Conversion of around 92 % of the substrate to ester could be realized using a concentration of lipase as low as 4.0 g dm^{-3} and in a relatively short time (26 h) at 35 °C, when a high substrate mole ratio of 2.5 was used.

Keywords: factorial design; surface response analysis; *Candida rugosa* lipase; flavor esters; esterification.

INTRODUCTION

Short chain aliphatic esters play a relevant role in the food industry as flavor and aroma constituents.[1] They are responsible for the particular fruity aroma or smell of a particular flower. Current processes for the production of esters consist

* Corresponding author. E-mail: zknez@tmf.bg.ac.yu
# Serbian Chemical Society member.
doi: 10.2298/JSC0812139K

of the esterification of a carboxylic acid with an alcohol in the presence of non-selective inorganic catalysts at high temperatures or extraction from natural sources. Esters extracted from plant materials are often either too scarce or expensive for commercial use while those produced by chemical synthesis are not considered as natural products. The replacement of inorganic catalysts by lipases in the synthesis of flavor esters avoids side product formation and is less polluting and energy consuming because of the mild conditions employed.[2] More importantly, the quality of the product of so enzyme-synthesized esters is normally better than the chemically-derived product due to the lower reaction temperature and avoidance of degradation products resulting from the strong acid-catalysis. Consequently, numerous attempts have been made to develop an efficient lipase system for the synthesis of food acceptable esters.[3–5]

Lipase from *Candida rugosa* (formerly *Candida cylindracea*) is an enzyme of considerable physiological significance and industrial potential in that it can catalyze numerous reactions, such as hydrolysis, transesterification, esterification, alcoholysis, acidolysis and aminolysis.[6–8] Several researchers have reported the synthesis of short-chain esters using free or immobilized lipase in non-aqueous, solvent-free or biphasic organic phase reaction systems.[3,9–11] In order to maximize the yield of the esters, serious attention was given to the optimization of the process parameters, the development of appropriate kinetic models of esterification and the monitoring and control of the water concentration in the reaction mixture. However, most of the studies were based on conventional one-at--a-time variation of parameters, which often do not demonstrate the interactive effects of the parameters.Moreover, the widely different substrates and reaction systems employed led to an enormous amount of quantitative data, which, however, cannot be directly compared. Thus, to make the enzymatic processes competitive, they should be studied and compared in a systematic way.

It was shown that the statistical designs could be very useful tool not only in optimizing esterification reactions, but also in explaining qualitatively and quantitatively the esterification behavior of the employed lipase.[12] In addition to reducing the number of experiments required for optimization, this technique enables the quantification of the individual effect of each factor and to investigate their possible interactions. An increasing number of results published in the field of ester synthesis in an organic solvent were based on experimental design.[13–15] Most of these studies were focused on the optimization of the conditions for the synthesis of ethanoic acid esters with immobilized lipase from *Rhizomucor miehei* as the catalyst. However, comparatively few have related to systematic studies of the synthesis of butanoic acid and 2-methyl propanoic acid esters with lipase from *C. rugosa*.[16]

The object of this study was to investigate the process conditions relevant for the synthesis of flavor esters aiming at a better control of the enzymatic process.

The results of previously reported investigations implied that the influence of reaction factors varies strongly with both substrate types. Each ester synthesis seems to be a specific problem. In this study, the synthesis of pentyl 2-methylpropanoate in isooctane was chosen as a model reaction because of its importance in the food industry. This short chain ester, which contributes to the natural aroma of fruits such as pineapple and banana, is widely used in the food industry for flavor enhancement. Lipase from *C. rugosa* was chosen for present work because of its commercial availability in large quantities at relatively low cost and the number of immobilization procedures developed with this enzyme.[17–19] Response surface methodology and 5-level-5-factor central composite rotatable design were performed to identify the factors that influence the ester synthesis and to verify whether any changes should be made in their settings to improve this reaction. In spite of the importance of pentyl 2-methylpropanoate, to the best of our knowledge, there are no reports about the use of *C. rugosa* for the synthesis of this ester in a non-aqueous system. Moreover, this is the first time that this system has been analyzed by the statistical approach at this level of detail.

EXPERIMENTAL

Materials

Commercial *Candida rugosa* lipase (EC 3.1.1.3), trizma buffer, thymolphthalein indicator solution and olive oil emulsion were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The lipase was a crude preparation with 10 % protein based on the Lowry method for protein assay.[20] 2-Methylpropanoic acid and *n*-pentanol were purchased from Merck (Darmstadt, Germany). 2,2,4-Trimethylpentane of p.a. grade, purchased from Merck (Darmstadt, Germany), was dried over 0.3 nm molecular sieves for at least 24 h prior to use and, as such, was regarded as nearly anhydrous. The solvents used in the analytical procedures, standards and other reagents were reagent grade and purchased either from Aldrich Chemical Co. (St. Louis, MO, USA) or Sigma Chemical Co. (St. Louis, MO, USA).

Lipase activity assay

The lipase activity was estimated by the standard olive oil emulsion method.[21] This activity assay was performed with reaction mixtures containing 3.0 cm^3 of Sigma lipase substrate, 1.0 cm^3 of trizma buffer, and 3.5 cm^3 of distilled water. Negative and positive controls were also studied. The positive control was the reaction mixture in which investigated solution or biocatalyst was added at the beginning of the reaction time, whereas the negative control was one with samples added just before titration with NaOH. The reaction mixtures were agitated and incubated for 30 min in a water bath at 37 °C. The formed fatty acids were quantified by titration with 0.050 M sodium hydroxide. The activities are expressed as international units (IU), where 1 IU is defined as the amount of enzyme required to produce 1 μmol min^{-1} of free fatty acid under the assay conditions (37 °C, pH 7.7). The determined activity of the lipase was 1.55 IU mg^{-1} enzyme.

Pentyl 2-methylpropanoate synthesis

The esterification reactions were performed in screw-capped 100 cm^3 flasks in 2,2,4-trimethylpentane. *n*-Pentanol and 2-methylpropanoic acid were added at different molar ratios followed by different amounts of water, according to the experimental design. The reaction mixture was then diluted up to the volume of 10 cm^3 with 2,2,4-trimethylpentane and incu-

bated on a shaker at 150 rpm and at different temperatures prior to the addition of the lipase. The various quantities of enzyme were added to the reaction mixture only after the correct temperature had been attained and samples were taken for analysis after 4, 15, 26, 37 and 48 h reaction time. Control experiments were also conducted without lipase under similar conditions.

Analysis

The reactions were monitored by determination of the residual acid content by titration against standard sodium hydroxide using phenolphthalein as the indicator and methanol as a quenching agent. The molar conversion was determined from the values obtained for the blank and the test samples. The reactions were also monitored by measuring the concentrations of the products by gas chromatography (model: Varian 3400) equipped with a Carbowax 20-M column (3.0 m length, 3.175 mm internal diameter) and a flame ionization detector (FID). Nitrogen was used as the carrier gas at a flow rate of 30 cm^3 min^{-1}. The column oven, injector and detector temperatures were at 100, 200, and 250 °C, respectively.[14] The reported percentage yield of ester was defined as the amount of ester produced to the amount of initial substrate in defect ((mol ester/mol initial substrate in defect)×100). The percentage esterification determined by both GC analysis and titration were found to be in good agreement.

Experimental design and analysis

A five-level-five-factor central composite design was employed in this study, requiring 32 experiments, which consisted of 16 factorial points, 10 axial points and 6 central points.[22] The variables and their levels selected for the study of the ester synthesis were: water content (0.0–2.0 % (w/v)); temperature (35–75 °C); enzyme concentration (1.0–5.0 g dm^{-3}); acid/alcohol mole ratio (1:2–5:2) and reaction time (4–48 h). These variables were chosen based on the results obtained in a preliminary study and are the most commonly used for modeling esterification reactions. In a preliminary study, the effects of reactant concentration on the initial rate of production of pentyl 2-methylpropanoate were investigated with the reactants added in stoichiometric proportions. It seems that the initial reaction rate increases rapidly with concentration up to about 0.50 mol dm^{-3} and thereafter becomes essentially constant between 0.50 and 0.75 mol dm^{-3} before dropping at higher concentrations (data not shown). This appears to be caused by inactivation of the lipase by the alcohol. In separate experiments in which an excess of 2-methylpropanoic acid was used, it was shown that the acid did not deactivate the enzyme in concentrations up to 1.25 mol dm^{-3}. Therefore, the effect of acid/alcohol mole ratio was investigated fixing the initial alcohol concentration at a lower value (500 mmol dm^{-3}) at different concentrations of acid.

The actual and coded settings of each of the five experimental factors are given in Table I. The experiments were run at random to minimize errors due to possible systematic trends in the variables. The ester yield was taken as the response variable. The design of experiments employed is presented in Table II. The data obtained were fitted to a second-order polynomial equation:

$$Y = \beta_{k0} + \sum_{i=1}^{5} \beta_{ki} X_i + \sum_{i=1}^{5} \beta_{kii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} \beta_{kij} X_i X_j \quad (1)$$

where Y is the response (ester yield in mol %), β_{k0},β_{ki}, β_{kii} and β_{kij} are regression coefficients for the intercept, linear, quadratic and interaction terms, respectively and X_i and X_j are independent variables. The coefficients of the response function and their statistical significance were evaluated by the method of least squares using Matlab software (version 6.5, Release 13, The MathWorks, Juc, Matick, MA, USA). Only the significant terms ($p \leq 0.05$) were considered for the final reduced model. The lack-of-fit test was used to determine whether the

constructed model was adequate to describe the obtained data. The goodness of fit of the model was evaluated by the determination of R^2 and r coefficients, complemented by a graphic plot of the values predicted by the model *vs*. the observed experimental values. High values of both R^2 and r suggest a good fit of the model to the experimental data. Response surfaces and contour plots were obtained using the fitted model by keeping two independent variables at a constant value while changing the other two variables. The regression analysis, statistical significance and response surfaces were also realized using Matlab software.

TABLE I. Coded and actual values of the variables for the design of the experiments

Variables	Coded levels of the variables				
	–2	–1	0	1	2
Water content, X_1 / % (w/v)	0	0.5	1.0	1.5	2.0
Temperature, X_2 / °C	35	45	55	65	75
Enzyme amount, X_3 /g dm^{-3}	1	2	3	4	5
Substrate mole ratio, X_4	1:2	1:1	3:2	2:1	5:2
Reaction time, X_5 / h	4	15	26	37	48

RESULTS AND DISCUSSION

Response surface analysis

The response surface methodology (RSM) is an optimization technique, which determines the optimum process condition by testing several variables simultaneously, uses special experimental designs to reduce the number of required determinations and measures several effects by objective tests. In this study, RSM and 5-level-5-factor central composite rotatable design were employed to optimize and understand the relationship between the important reaction parameters in the lipase-catalyzed synthesis of pentyl 2-methylpropanoate in a non-aqueous system.

The data showing the predicted and experimental yields of ester for the 32 experiments of the statistical design are given in Table II. According to this study, the maximum ester yield can be obtained at a low temperature, low level of water content and high levels of lipase concentration, initial acid/alcohol mole ratio, and reaction time. Among the various syntheses, the greatest molar conversion (96.8 %) was achieved in run No. 13 (water content of 0.5 %, 45 °C, enzyme concentration of 4 g dm^{-3}, substrate mole ratio 2:1, 37 h), while the smallest conversion (only 11.85 %) was achieved in run No. 26 (water content of 1.0 %, 55 °C, enzyme concentration 3 g dm^{-3}, substrate mole ratio 3:2, 4 h).

A statistical analysis was performed on the experimental data, whereby the main effects and interaction effects of the variables were estimated. Both the *t*-test and *p*-value statistical parameters were used to confirm the significance of factors studied. The effects of the parameters on the ester synthesis and their significance are shown in the Pareto chart of effects (Fig. 1). It seems that the most relevant variables for the ester synthesis are reaction time and enzyme concentration with estimated effects of 13.00 and 11.61, respectively. The effects of substrate mole

TABLE II. Experimental setup for five-level, five-factor surface response design and the experimental data

Run No.	Water content, X_1 / %	Temperature X_2 / °C	Enzyme concentration X_3 /g dm^{-3}	Substrate mole ratio, X_4	Reaction time X_5 / h	Experimental
1	1	1	1	1	1	56.20
2	1	1	1	–1	–1	27.05
3	1	1	–1	1	–1	30.80
4	1	1	–1	–1	1	15.40
5	1	–1	1	1	–1	49.20
6	1	–1	1	–1	1	62.90
7	1	–1	–1	1	1	56.50
8	1	–1	–1	–1	–1	13.80
9	–1	1	1	1	–1	52.90
10	–1	1	1	–1	1	36.30
11	–1	1	–1	1	1	36.70
12	–1	1	–1	–1	–1	14.30
13	–1	–1	1	1	1	96.80
14	–1	–1	1	–1	–1	30.00
15	–1	–1	–1	1	–1	29.30
16	–1	–1	–1	–1	1	42.60
17	2	0	0	0	0	24.00
18	–2	0	0	0	0	36.22
19	0	2	0	0	0	13.05
20	0	–2	0	0	0	33.45
21	0	0	2	0	0	76.57
22	0	0	–2	0	0	14.25
23	0	0	0	2	0	61.75
24	0	0	0	–2	0	29.45
25	0	0	0	0	2	80.92
26	0	0	0	0	–2	11.85
27[a]	0	0	0	0	0	48.30
28[a]	0	0	0	0	0	29.10
29[a]	0	0	0	0	0	28.42
30[a]	0	0	0	0	0	41.02
31[a]	0	0	0	0	0	31.35
32[a]	0	0	0	0	0	35.92

[a]Center point

ratio, temperature, and temperature–reaction time interaction were also signifycant ($p < 0.05$). It appears that while the substrate mole ratio has a positive effect (8.87), temperature and reaction time–temperature interaction have a significant negative influence on the ester yield (–6.93 and –7.09, respectively) which is in agreement with thermal stability data for this lipase in non-aqueous medium.[10,23,24] The results also indicate the importance of working at high levels of enzyme concentration, reaction time and substrate mole ratio. Due to the bars that extend beyond the vertical line on the plot which correspond to effects that are stati-

stically significant at the 95 % confidence level, quadratic terms of incubation time, mole ratio and enzyme concentration were also significant. Interestingly, the effect of the water content on the conversion was not significant and could be neglected in the range tested. Perhaps, this is because a water content in this range was sufficient to preserve the catalytic conformation of the enzyme and the lipase itself contained sufficient water to maintain its activity. In general, it was observed that only a very small amount of water was required to successfully employ enzymes in organic solvents.[10] However the optimal level of water should be determined for each particular reaction system. In the present experimental setup, the water content did not significantly influence the ester yield. Therefore, the added water was constant at 0 level (1 %) in the following discussion.

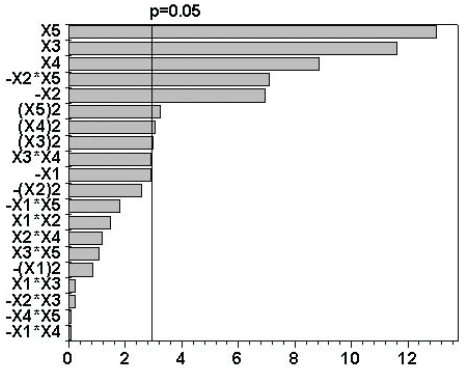

Fig. 1. Pareto diagram of the effect of the reaction parameters on the ester synthesis and their significance calculated from the experimental design. For abbreviations, see Table I.

The multiple regression coefficients, obtained by employing a least squares technique to predict a second-order polynomial model (Eq. (1)) for ester yield (Y, %), after backward elimination, their significance (student's t-test and p-values) and the results of the statistical analysis are summarized in Table III. According to the results of the student's t-test, four linear coefficients and one cross-product term had a highly significant effect at the 99 % confidence level. Three quadratic terms were also significant ($p < 0.05$). The quadratic term of temperature and nine cross-product terms corresponding to X_1 with X_2, X_3, X_4 and X_5, X_2 with X_3 and X_4, X_3 with X_4 and X_5 and X_4 with X_5 were found to be insignificant ($p > 0.05$). The final response equation obtained after eliminating the insignificant terms is as follows:

$$Y = 31.959 - 6.935X_2 + 11.767X_3 + 9.019X_4 + 12.849X_5 - 7.093X_2X_5 + \\ + 3.207\,X_3^2 + 3.254\,X_4^2 + 3.451\,X_5^2 \quad (2)$$

The fit of the model was checked by the R^2 values, which was calculated to be 0.912, indicating that 91.2 % of the variability in the response could be explained by the model. The model also showed statistically insignificant lack of fit, as is evident from the lower calculated F value (1.06) than the theoretical $F_{0.05}$ value (4.58) at the 5 % level. The plot of experimental values of ester yield

(%), *versus* those calculated from the above equation, indicated a good fit (Fig. 2), with a correlation coefficient, r of 0.944. Overall, these results revealed good agreement between the predicted and experimental values, implying that the empirical model derived from RSM can be used to adequately describe the relationship between the factors and the response in the lipase-catalyzed synthesis of pentyl 2-methylpropanoate.

TABLE III. Regression coefficients (β) and significance (student's t-test and p-values) of the predicted second-order polynomial model for the response (Y) after backward elimination and the results of the statistical analysis

Factors	Coefficient (β)	Standard error	t-Values	p-Values
Average	31.9595	±3.11	11.31	0.0000
Temperature, X_2	–6.9348	±1.68	–4.23	0.0027[a]
Lipase concentration, X_3	11.7673	±1.68	6.93	0.0002[a]
Substrate mole ratio, X_4	9.0193	±1.68	5.29	0.0009[a]
Reaction time, X_5	12.8494	±1.68	7.76	0.0001[a]
X_2X_5	–7.0930	±2.32	–3.50	0.0064[a]
X_3^2	3.2070	±1.45	2.06	0.0422[b]
X_4^2	3.2539	±1.44	2.10	0.0404[b]
X_5^2	3.4507	±1.45	2.23	0.0335[b]

Results of the statistical analysis

Source of variation	Sum of square	Degrees of freedom	Mean square	F-test
Regression	1455.8	23	63.3	–
Lack of fit	1153.2	18	64.1	1.06
Pure error	302.6	5	60.5	–

[a]$p < 0.01$; [b]$p < 0.05$

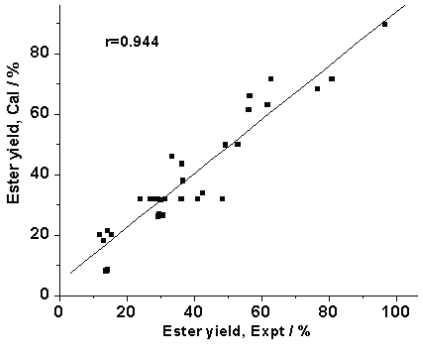

Fig. 2. Correlation of the calculated *versus* the experimental values for the synthesis of pentyl 2-methylpropanoate catalyzed by lipase from *C. rugosa*.

Influence of process conditions on the molar conversion

The influence of the variables, reaction temperature, enzyme concentration, substrate mole ratio and reaction time on the yield of ester is discussed using the statistical model shown by Eq. (2).

Temperature showed an interactive effect with the reaction time of esterification. The shape of the three-dimensional surface, representing yield of ester *versus* temperature and reaction time, is shown in Fig. 3a. It appears that the

surface is smooth showing an increase/decrease in one axis and a decrease/ /increase in the other axis, which reflects that the temperature may affect the reaction rate in opposite ways. Specifically, as the temperature increases, the expected increase in reaction rate resulting from more productive molecule collisions per unit time is offset by the increasing rate of enzyme denaturation. The effect of temperature on the ester synthesis during the initial period was observed to follow Arrhenius law (between 298 and 338 K) with an activation energy of 18.34 kJ mol^{-1}, which is typical for enzymatic reactions occurring in a reaction--limited regime.[25] At intermediate and high levels of reaction time, however, a different behavior was observed as the surface decreased with increasing reaction temperature. This could be the result of a negative temperature–reaction time interaction, probably caused by thermal deactivation of the enzyme. The maximum yield of ester could be obtained when working at low temperatures and a high level of reaction time. The result suggests that the *C. rugosa* lipase, like some other lipases such as Novozym SP 435 from *Candida antarctica*[26,27] or porcine pancreatic lipase,[12] was inactivated when it was subjected to a high temperature for a long period under non-aqueous conditions.

Another important parameter affecting the economic feasibility of the process is the acid to alcohol mole ratio. Figure 3b shows the predicted percentage of esterification as a function of the substrate molar ratio at different temperatures for an enzyme concentration of 3 g dm^{-3} (4.5 IU cm^{-3}) and an incubation period of 26 h. It seems that while the temperature exerted a negative influence, excess of acid had a significant positive effect on the ester yield, indicating the importance of using an excess of acid over the stoichiometric amount for the maximum conversion to ester. Minimum esterification was observed at a substrate mole ratio of 0.8 at 55 °C (11 %) and a maximum esterification was observed at a substrate ratio of 2.5 at 35 °C (≈ 77 %). The beneficial effect of excess acyl donor was also observed for the synthesis of short-chain esters using microbial lipases by several authors. For example, studying the synthesis of ethyl butanoate with the same lipase as the catalyst, Chen[9] verified that the mole ratio between ethanol and butanoic acid was a critical factor for attaining a high yield of ethyl butanoate, requiring an amount of butanoic acid of the order of 3.3 times that of ethanol. Yadav and Lathi[27] also found that there was an increase in the reaction rate with increasing amount of 2-methylpropanoic acid using Novozym SP 435 lipase during the synthesis of butyl 2-methylpropanoate. The possible explanation is related to reaction mechanism. Lipase is known to catalyze esterification through an acyl-intermediate formed between the fatty acid substrate and the enzyme. Free enzyme can either bind the fatty acid to produce this intermediate or the ester product. In an excess of fatty acid, most of the enzyme is found in the acylated form, preventing it from binding the product. In addition, a higher concentration of free 2-methylpropanoic acid in the reaction system was

beneficial for the incorporation of acid from the view of reaction equilibrium, but excessive free fatty acid could also result in substrate inhibition.[10,25,28] In the present experimental setup, the substrate mole ratio had a positive influence on the ester yield and it was found that the acid did not deactivate the catalyst at concentrations up to 1.25 mol dm^{-3}.

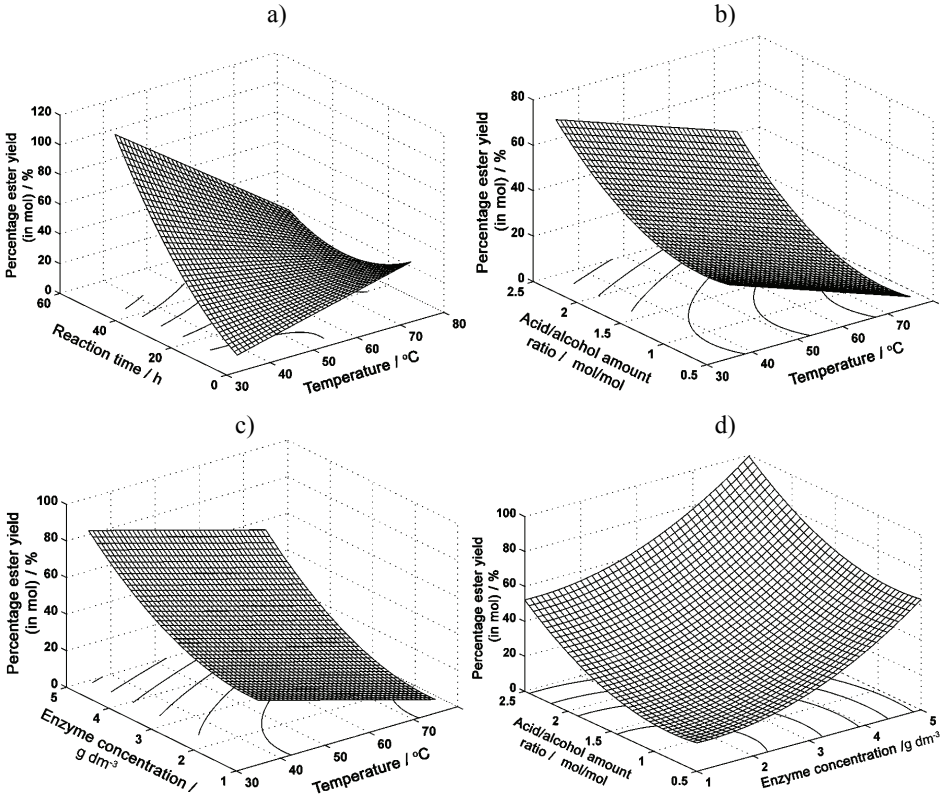

Fig. 3. Correlation of the calculated *versus* the experimental values for the synthesis of pentyl 2-methylpropanoate catalyzed by lipase from *C. rugosa*.

The response surface plot for the predicted values for the yield of ester *versus* temperature and catalyst concentration after 26 h at a fixed substrate mole ratio of 1.5 are shown in Fig. 3c. It appears that predicted yield of ester increased with increasing enzyme concentration at all temperatures. For an illustration, at 55 °C, the response varied from 21.2 to 68.3 % on increasing the enzyme concentration from 1–5 g dm^{-3} (1.5–7.5 IU cm^{-3}). A similar behavior was observed during the *Rhizopus* lipase-catalyzed synthesis of 3-methylbutyl butanoate, since an increase in yield by 32.2 % was recorded when the amount lipase was changed from 1 to 10 %.[29] Therefore, to maximize the ester yield, the enzyme concentration must be kept at the highest tested levels.

Similar trends were observed for the interaction of both enzyme concentration and substrate mole ratio *versus* reaction time. However, the most interesting result of the part of study focused on the statistically analyzed influence of enzyme concentration and mole ratio in a 3-dimensional graph (Fig. 3d). In addition, the contour plot could also indicate the desirable combination of variables, which can be selected by the manufacturer, because there were several optimal combinations available to obtain the highest ester yield (Fig. 4). The ester production is represented by a concave surface described by a second order polynomial with a minimum at an enzyme concentration of about 1.2 g dm^{-3} (3.6 IU cm^{-3}) for a mole acid/alcohol ratio equal to 0.8. Figure 4 shows the contour plot for the predicted values for the yield of ester *versus* catalyst concentration and substrate mole ratio after 26 h at 35 °C. The results indicate that high yields are possible with small amounts of enzyme when high substrate mole ratio levels are used,

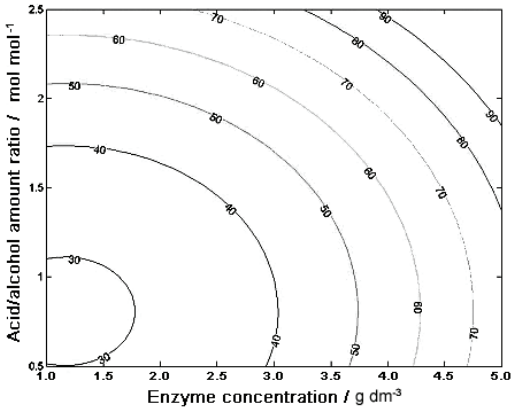

Fig. 4. Contour plot for the yield of ester as a function of enzyme concentration and substrate mole ratio after 26 h at 35 °C.

which is beneficial from the economic viewpoint since the cost of enzyme is usually higher than that of substrates. In general, for enzymatic esterification reactions, the lipase concentrations required to achieve higher yields of esters are often too high and the reaction times relatively too long for industrial application. Welsh *et al.*[4] reported 75.8 % conversion in 48 h with 2 % native lipase from *C. cylindracea* at 0.050 M substrate (the E/S ratio was 400 g mol^{-1}). Chowdary *et al.*[30] reported an 85 % conversion of 3-methylbutanoic acid to 3-methylbutyl 3-methylbutanoate with 0.50 M acid concentration and 1.0 % enzyme concentration during an incubation time of 144 h in *n*-hexane (the E/S ratio was 20), by using Lipozyme IM-20 lipase from *R. miehei*. In this study, it was shown that a high conversion of 91.9 % could be achieved at 0.50 M alcohol concentration (substrate in deficit), using amounts of enzyme as low as 4.0 g dm^{-3} and in a relatively short time (26 h) at 35 °C at a fixed acid/alcohol mole ratio of 2.5, as can be inferred from the contour plot in Fig. 4. Namely, under this condition (E/S ratio of $\approx$ 8 g mol^{-1}), a rather high ester concentration of around 70 g dm^{-3} was

achieved, which was in the proximity of results previously reported in related studies[28,30] or much higher.[4,5,31] The feasibility of the ester synthesis by *C. rugosa* lipase under solvent-free condition was also explored, and a reasonably high yield of esters (62 %) was achieved under optimal conditions (data not shown).

CONCLUSIONS

The aim of this work was to evaluate the performance of lipase from *C. rugosa* in the synthesis of pentyl 2-methylpropanoate using a reaction system of interest from an industrial point of view. A surface response methodology based on CCRD design was employed to study the effects of the five most important factors influencing the yield of ester. An ester yield response equation was obtained, making it possible to predict the operating conditions required to obtained well--defined amounts of the ester. It seems that the lipase concentration, reaction time and substrate mole ratio have positive influences on the ester synthesis while the temperature and reaction time–temperature interaction have negative influences on the process. It appears that high yields of esters are possible with small amounts of enzyme when high substrate mole ratios are used, which is beneficial from the economic viewpoint. These findings should stimulate the application of such lipase-catalyzed reactions for the preparation of food acceptable short chain esters. Further studies should be concentrated on improvement of the lipase stability by its immobilization and extension of its application to other non-aqueous and solvent-free reaction systems.

Acknowledgements. The authors thank Ministry of Science and Technological Development of the Republic of Serbia for the financial support (Project: TR-20064).

ИЗВОД

СИНТЕЗА МИРИСНИХ ЕСТАРА КАТАЛИЗОВАНА ЛИПАЗАМА У НЕВОДЕНОЈ СРЕДИНИ: ОПТИМИЗАЦИЈА ПРИНОСА ПЕНТИЛ-2-МЕТИЛПРОПАНОАТА СТАТИСТИЧКОМ АНАЛИЗОМ

ЗОРИЦА КНЕЖЕВИЋ-ЈУГОВИЋ[1], ДЕЈАН БЕЗБРАДИЦА[1], ЖИВАНА ЈАКОВЉЕВИЋ[2], СУЗАНА БРАНКОВИЋ-ДИМИТРИЈЕВИЋ[1] и ДУШАН МИЈИН[1]

[1]*Технолошко–металуршки факултет, Универзитет у Београду, Карнегијева 4, 11120 Београд и* [2]*Машински факултет, Универзитет у Београду, Краљице Марије 16, 11120 Београд*

У раду су испитани утицаји различитих процесних параметара на синтезу пентил-2-метилпропаноата катализовану липазом из *Candida rugosa.* У циљу оптимизације ензимске синтезе естара примењена је методологија одзивних површина у складу са одабраним централним композиционим ротабилним планом (пет фактора на пет нивоа). Испитани су утицаји процесних параметара на принос естра у следећим интервалима: почетног садржаја воде (0,0–2,0 %), температуре (35–75 °C), концентрације ензима (1,0–5,0 g dm^{-3}), почетног молског удела супстрата (1:2–5:2) и реакционог времена (4–48 h). Добијен је адекватан математички модел на основу кога се може предвидети понашање система у функцији ових пет фактора. Показано је да концентрација ензима, почетни молски однос супстрата и реакционо време имају нај-

већи утицај на процес, док садржај воде не утиче значајно на принос естра. Под оптималним условима ензимске синтезе остварен је принос естра око 92 %.

(Примљено 19. марта, ревидирано 3. јуна 2008)

REFERENCES

1. M. Liaquat, R. K. Owusu, *Food Chem. Toxicol.* **65** (2000) 295
2. U. Krings, R. G. Berger, *Appl. Microbiol. Biotechnol.* **49** (1998) 1
3. F. W. Welsh, R. E. Williams, *Enzym. Microb. Technol.* **12** (1990) 743
4. F. W. Welsh, R. E. Williams, K. H. Dawson, *J. Food Sci.* **55** (1990) 1679
5. H. Razafindralambo, C. Blecker, G. Lognoy, M. Marlier, J. P. Wathlet, M. Severin, *Biotechnol. Lett.* **16** (1994) 247
6. S. Benjamin, A. Pandey, *Yeast* **14** (1998) 1069
7. D. Bezbradica, I. Karalazić, N. Ognjanović, D. Mijin, S. S. Marinković, Z. Knežević, *J. Serb. Chem. Soc*. **71** (2006) 31
8. Z. Knežević, S. S. Marinković, L. Mojović, *Appl. Microbiol. Biotechnol.* **49** (1998) 267
9. J. P. Chen, *J. Ferment. Bioeng.* **82** (1996) 404
10. G. Carta, J. L. Gainer, A. H. Benton, *Biotechnol. Bioeng*. **37** (1991) 1004
11. D. Bezbradica, D. Mijin, S. S. Marinković, Z. Knežević, *J. Mol. Catal. B: Enzym*. **38** (2006) 11
12. B. Manohar, S. Divakar, *Process Biochem.* **39** (2004) 847
13. C. J. Shieh, S. W. Chang, *J. Agric. Food Chem.* **49** (2001) 1203
14. S. H. Krishna, B. Manohar, S. Divakar, S. G. Prapulla, N. G. Karanth, *Enzym. Microb. Technol.* **26** (2000) 131
15. B. Manohar, S. Divakar, *World J. Microbiol. Biotechnol.* **18** (2002) 745
16. I. L. Shih, S. H. Hung, F. Y. Chen, H. Y. Ju, C. J. Shieh, *Food Chem.* **100** (2007) 1223
17. S. H. Chiou, W. T. Wu, *Biomaterials* **25** (2004) 197
18. Z. Knežević, L. Mojović, B. Adnađević, *J. Serb. Chem. Soc*. **63** (1998) 257
19. J. M. Moreno, J. V. Sinisterra, *J. Mol. Catal.* **93** (1994) 357
20. O. H. Lowry, N. J. Resebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193** (1951) 265
21. N. Tietz, E. Fiereck, *Clin. Chim. Acta* **13** (1966) 352
22. G. E. P. Box, W. G. Hunter, J. S. Hunter, *Statistics for experimenters: an introduction to design, data analysis and model building*, Wiley, New York, 1978, p. 653
23. F. M. Gomes, E. B. Pereira, H. F. Castro, *Biomacromolecules* **5** (2004) 17
24. Z. Knežević, N. Milosavić, D. Bezbradica, Ž. Jakovljević, R. Prodanović, *Biochem. Eng. J.* **30** (2006) 269
25. A. Zaidi, J. L. Gainer, G. Carta, A. Mrani, T. Kadiri, Y. Belarbi, A. Mir, *J. Biotechnol.* **93** (2002) 209
26. J. M. Rodriguez-Nogales, E. Roura, E. Contreras, *Process Biochem.* **40** (2005) 63
27. G. D. Yadav, P. S. Lathi, *Biochem. Eng. J.* **16** (2003) 245
28. S. H. Krishna, S. G. Prapulla, N. G. Karanth, *J. Ind. Microbiol. Biotechnol.* **25** (2000) 147
29. G. A. Macedo, G. M. Pastore, M. I. Rodrigues, *Process Biochem.* **39** (2004) 687
30. G. V. Chowdary, M. N. Ramesh, S. G. Prapulla, *Process Biochem.* **36** (2000) 331
31. G. Langrand, N. Rondot, C. Triantaphylides, J. Baratti, *Biotechnol. Lett.* **18** (1990) 581.