Journal of the Serbian Chemical Society

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

J. Serb. Chem. Soc. *73* (2) 147–156 (2008)
JSCS–3697

UDC 66.011+665.75.000.57.002.2:577.15
Original scientific paper

Optimization of the production of biodiesel by a commercial immobilized lipase in a solvent-free system using a response surface methodology

NEVENA OGNJANOVIĆ, DEJAN BEZBRADICA[#] and ZORICA KNEŽEVIĆ*[#]

Faculty of Technology and Metallurgy, Karnegijeva 4, 11000 Belgrade, Serbia

(Received 14 March, revised 2 July 2007)

Abstract: Response surface methodology was used for the evaluation of the effects of various factors on the synthesis of biodiesel catalyzed with immobilized lipase from *Rhizomucor miehei* in a solvent-free system. The production of biodiesel was optimized and model response equations were obtained, enabling the prediction of biodiesel production from the values of the four main factors. It would seem that the reaction temperature and the amount of water predominantly determined the conversion process while the methanol/oil molar ratio had no significant influence on the reaction rate. The temperature and amount of water showed negative interactive effects on the observed reaction rate per amount of enzyme. However, there were no significant interactions among the other variables according to the test of statistical significance. The highest yield of 10.15 mol kg^{-1} enzyme was observed at 45 °C with a 6:1 methanol to oil molar ratio and with no added water in the system.

Keywords: lipases from *Rhizomucor miehei*; biodiesel; response surface methodology.

INTRODUCTION

Biodiesel (fatty acid methyl esters), used as alternative diesel engine fuel, has become increasingly important due to the diminishing petroleum reserves and the environmental consequences of exhaust gases from petroleum-fueled engines. Biodiesel has several advantages: it comes from renewable sources and, as such, it does not contribute to new carbon dioxide emission, it is biodegradable, its combustion products have reduced levels of particulates, carbon monoxide, sulfur oxides, hydrocarbons and soot.[1]

Industrially, biodiesel is produced by the methanolysis of waste oil using alkaline catalysts.[2] There are several drawbacks to this conventional chemical production, such as the cost of the refined feedstock, the recovery of glycerol, the

---

[#] Serbian Chemical Society member.

*Corresponding author. E-mail: zknez@tmf.bg.ac.yu

doi: 10.2298/JSC0802147O

necessity to exclude the catalyst, undesirable side reactions, removal of inorganic salts and high reaction temperature. To overcome such drawbacks, there have been some attempts to develop and optimize methods for the production of biodiesel from a variety of lipids *via* different chemical or enzymatic catalysis.[3,4] Although the concept of biodiesel production without the use of any catalyst in supercritical methanol seems to be a very promising approach, the consumption of methanol was relatively high and the method required a high temperature of 350 °C and a pressure of 45 MPa, which would increase the production costs.[5] Due to the high-energy costs of chemical processes, the application of lipase in the oleochemical industry has become more attractive. The employment of lipases as biocatalysts allows mild reaction conditions and easy recovery of glycerol without purification or chemical waste production.[6,7]

Considerable research has been performed on the transesterification of vegetable oils and animal fats with different acyl acceptors using various free or immobilized lipases as catalysts in organic solvents or solvent-free systems.[8–10] However, the majority of enzymatic syntheses of biodiesel were performed in organic solvents, which are not suitable from the application viewpoint because of the toxicity and flammability of the solvent, damaging effects on the environment and consequential requirement for solvent removal. Thus, to enable enzymatic processes to be competitive, enzymatic solvent-free systems have been developed.[7,9–13]

It was shown that immobilized *Rhizomucor miehei* lipase (Lipozyme RM-IM) is a suitable catalyst for the alcoholysis of several oils and fats with primary alcohols in *n*-hexane.[14] Alcoholysis with relatively long-chain linear alcohols proceeds efficiently even in an organic solvent-free system but organic solvent-free methanolysis typically does not give high conversions.[15] The main obstacle for using methanol as a substrate is its detrimental effect on the lipase activity, as several studies reported that a high methanol concentration could lead to serious inactivation of lipase.[11,15] In order to circumvent this limitation, a three-step batch methanolysis process was developed and conversions of around 80 % were achieved even without solvent.[8] Additionally, the possibility of increasing the yield of methyl esters by a surplus of methanol was studied.[8,9] However, like most studies dealing with enzymatic methanolysis of vegetable oils, the experiments were based on the conventional one-at-a-time variation of the parameters, which often does not demonstrate the interactive effects of the parameters. Considering the high demand and benefits, improved knowledge of the relationships between the important transesterification variables and biodiesel yield is necessary to develop an efficient lipase catalytic process.

In this study, the effects of various factors on the methanolysis of sunflower oil by a commercial lipozyme lipase from *Rhizomucor miehei* was studied in a solvent-free system. Response surface methodology (RSM) and 5-level-4-factor central composite rotatable design (CCRD) was performed to evaluate the effect of each of the analyzed factors, as well as the interactive effects of these factors on the synthesis of the methyl ester. This kind of experimental design reduces the number of experiments to be preformed, thus reducing the costs, and gives knowledge of the interaction among the variables within the studied range. Although an increasing number of results published in the field of biodiesel synthesis in an organic solvent are based on experimental design,[16,17] a systematic study related to the transesterification of vegetable oil with the immobilized *R. miehei* lipase in a solvent-free system has not hitherto been performed.

EXPERIMENTAL

Materials

Commercial lipase from *R. miehei* immobilized on a macroporous anion exchange resin (Lipozyme RM-IM) was kindly gifted by Novo Nordisk (Bagsvaerd, Denmark). Refined sunflower oil "Sunce" (Sunce a.d., Sombor) and methanol (purity > 99.8 %, Sigma, USA) were used as the reactants for the enzymatic reaction. Methyl myristate (Fluka, Switzerland) was used as the internal standard. All other chemicals were of reagent-grade.

Alcoholysis reaction

The synthesis was performed in 100 ml stoppered flasks, as a three-step methanolysis process. The first portion of methanol and the total amount of oil were added at the start of the reaction; the second portion of methanol was added after 10 h, while the third portion was added after 25 h, according to previously obtained results.[18] The total reaction time was 50 h. Appropriate amounts of enzyme, methanol and water were added in the reaction media according to the experimental design (Table I). The amount of enzyme varied from 1–5 %, water 0–10 % and substrate molar ratio 3–9. The mixture was agitated on a shaker at 150 rpm at different temperatures (25–65 °C).

Analysis of the samples

The contents of methyl ester in the reaction mixture were quantified using a GS Varian 3400 instrument with a fused silica capillary column (5 m×0.53 mm×0.5 μm). The column temperature was held at 60 °C for 1 min, then heated to 110 °C at 50 °C min^{-1}, heated again to 170 °C at 4 °C min^{-1} and then raised to 340 °C at 20 °C min^{-1} and maintained at this temperature for 45 min. The temperatures of the injector and detector were set at 300 °C and 340 °C, respectively. Methyl myristate served as the internal standard.

Experimental design and statistical analysis

The experiments were conducted employing a central composite design, which aids the investigation of linear, quadric and cross product effects of four factors, each varied at five levels.[19] The factors studied were enzyme concentration, water concentration, methanol to oil molar ratio and temperature during the transesterification reaction. The assay conditions for the reaction parameters were taken at zero level (center point), level one (+1 and −1) and level two (+2 and −2) (Table I). The design of the experiments employed is also presented in Table I. For data evaluation, response surface methodology (RSM) was used, and a second order polynomial equation was tested (for both response 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. 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 Fisher test was used to determine the adequacy of the model and the Student distribution to evaluate the significance of the coefficients.

TABLE I. The obtained results of various runs of the central composite design with coded and actual values of variables (numbers in parenthesis represent coded independent variable levels)

Run	Temperature °C	Enzyme amount %	Content of water %	Substrate molar ratio $n_{\text{methanol}}/n_{\text{oil}}$	Rate of biodiesel production mmol h^{-1}	Rate of biodiesel production per enzyme amount mmol h^{-1} g^{-1}
1	35 (–1)[a]	2 (–1)	2.5(–1)	4.5 (–1)	0.0882	0.147
2	55 (1)	2 (–1)	2.5(–1)	4.5 (–1)	0.0517	0.0862
3	35 (–1)	4 (1)	2.5(–1)	4.5 (–1)	0.156	0.130
4	55 (1)	4 (1)	2.5(–1)	4.5 (–1)	0.139	0.115
5	35 (–1)	2 (–1)	7.5(1)	4.5 (–1)	0.110	0.183
6	55 (1)	2 (–1)	7.5(1)	4.5 (–1)	0.0369	0.0615
7	35 (–1)	4 (1)	7.5(1)	4.5 (–1)	0.131	0.109
8	55 (1)	4 (1)	7.5(1)	4.5 (–1)	0.0636	0.0530
9	35 (–1)	2 (–1)	2.5(–1)	7.5 (1)	0.0535	0.0891
10	55 (1)	2 (–1)	2.5(–1)	7.5 (1)	0.139	0.231
11	35 (–1)	4 (1)	2.5(–1)	7.5 (1)	0.109	0.0906
12	55 (1)	4 (1)	2.5(–1)	7.5 (1)	0.0617	0.0514
13	35 (–1)	2 (–1)	7.5(1)	7,5 (1)	0.0603	0.101
14	55 (1)	2 (–1)	7.5(1)	7,5 (1)	0.0316	0.0527
15	35 (–1)	4 (1)	7.5(1)	7,5 (1)	0.132	0.110
16	55 (1)	4 (1)	7.5(1)	7,5 (1)	0.0496	0.0413
17	25 (–2)	3 (0)	5(0)	6 (0)	0.152	0.169
18	65 (2)	3 (0)	5(0)	6 (0)	0.0746	0.0828
19	45 (0)	1 (–2)	5(0)	6 (0)	0.0318	0.106
20	45 (0)	5 (2)	5(0)	6 (0)	0.169	0.113
21	45 (0)	3 (0)	0(–2)	6 (0)	0.183	0.203
22	45 (0)	3 (0)	10(2)	6 (0)	0.0617	0.0685
23	45 (0)	3 (0)	5(0)	3 (–2)	0.0474	0.0527
24	45 (0)	3 (0)	5(0)	9 (2)	0.0966	0.107
25	45 (0)	3 (0)	5(0)	6(0)	0.0474	0.0527
26	45 (0)	3 (0)	5(0)	6 (0)	0.0515	0.0573
27	45 (0)	3 (0)	5(0)	6 (0)	0.0318	0.0353
28	45 (0)	3 (0)	5(0)	6 (0)	0.0978	0.109
29	45 (0)	3 (0)	5(0)	6 (0)	0.0431	0.0480
30	45 (0)	3 (0)	5(0)	6 (0)	0.0882	0.0980

[a] The numbers in parenthesis represent coded independent variable levels

RESULTS AND DISCUSSION

In this study, response surface methodology was used to optimize and understand the relationships between the important reaction parameters in the lipase-catalyzed transesterification of sunflower oil with methanol in a solvent-free system. The objective was to obtain basic information concerning the relationships between these variables and the response (the reaction rate), aiming at a better control of the enzymatic process.

Based on the experimental results, two models were obtained. The first one presents the influence of individual and interactive effects on the reaction rate and the second one presents the same effects on the reaction rate per unit amount of enzyme. The second model allows the economic efficiency of the process to be optimized, since the cost of the enzyme represents a significant constituent of the overall costs of the process.

Statistical analysis was performed on the experimental results and the main and interaction effects of the variables were estimated. The amount of the enzyme had the largest influence on the reaction rate. Temperature and the amount of water had the next largest linear effects. The methanol to oil molar ratio did not significantly influence the production of biodiesel. An insignificant factor, but with a very high value, was the interactive effect between temperature and water content.

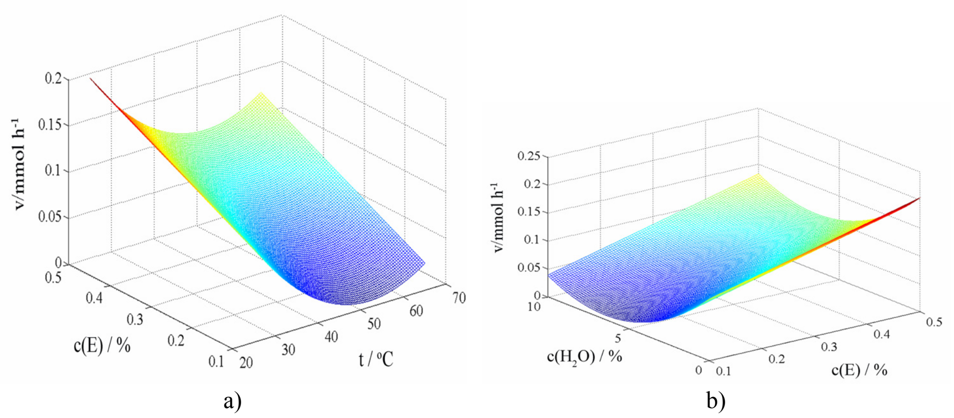
After eliminating the insignificant terms based on the composite rotatable design, a second order model (Eq. 1) was obtained with three significant parameters, without interactions:

$$Y_1 = 0.0596 - 0.0180x_1 + 0.0233x_2 - 0.0181x_3 + 0.0131\,x_1^2 + 0.0154\,x_3^2 \quad (1)$$

where Y_1 (mmol h^{-1}) is the rate of biodiesel production.

The response surface and contour plots of the predicted reaction rate as a function of process variables obtained from RSM analysis are presented in Fig. 1. Over the entire investigated range of enzyme concentration, the yield of the transesterification increased with increasing amount of enzyme in the reaction mixture (Figs. 1a and 1b). On the other hand, the effects of reaction temperature and amount of added water were described with quadratic equation with minimum values. The minimum rate of biodiesel production was achieved at 52 °C and 6.7 % of added water, while high rates were observed at high water content and a low temperature or at a low water content and a high temperature (Fig. 1c).

The transesterification reactions were performed over a wide range of temperatures, *i.e.*, from 25 to 65 °C. The response surface plot (Fig. 1a) clearly shows that increasing the temperature first led to a steep decrease of the rate of production of biodiesel until 45 °C. Further increasing of the temperature resulted in a slight increase of the rate of synthesis. The highest activity in examined temperature range was observed at the lowest applied temperature of 25 °C. The highest activity of the immobilized *R. miehei* lipase at the lowest employed temperature suggests that the lipase, like some other lipases, such as that from *Psendomonas cepacia*,[17] *Candida rugosa*[20] or porcine pancreatic lipase,[21] was inactivated when it was subjected to a higher temperature for a long period.



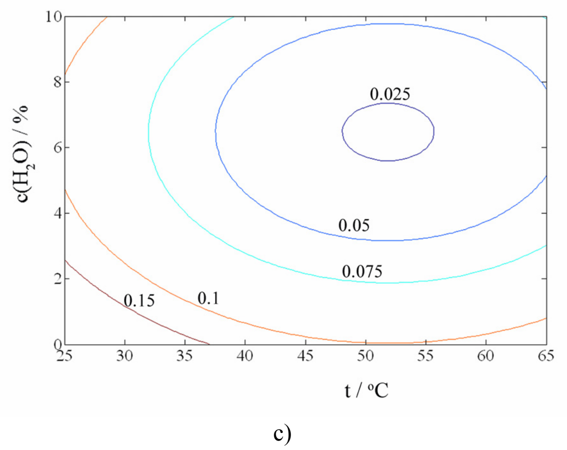


Fig. 1. a) The response surface of the reaction rate at different levels of enzyme concentration and temperature; b) the response surface of the reaction rate at different levels of water amount and enzyme concentration; c) contour plot of the reaction rate at different levels of water amount and temperature.

It is clear that significant parameters in the second model are temperature, water content and the interactive effect between temperature and water. From the experimental data and after eliminating the insignificant parameters, for the analysis of the factors on the reaction rate per unit amount of enzyme, the second order model (Eq. 2) with two significant parameters together with their interaction was obtained:

$$Y_2 = 0.0714 - 0.0198x_1 - 0.0227x_3 + 0.0163\,x_1^2 + 0.0191\,x_3^2 - 0.0219x_1x_3 \qquad (2)$$

where Y_2 (mmol h^{-1} g^{-1}) is the rate of biodiesel production per unit amount of enzyme.

The effect of both significant parameters on the specific rate of biodiesel production is illustrated in the response surface plot given in Fig. 2, from which it can be observed that high yields were obtained at low temperature and high water content or *vice versa*.

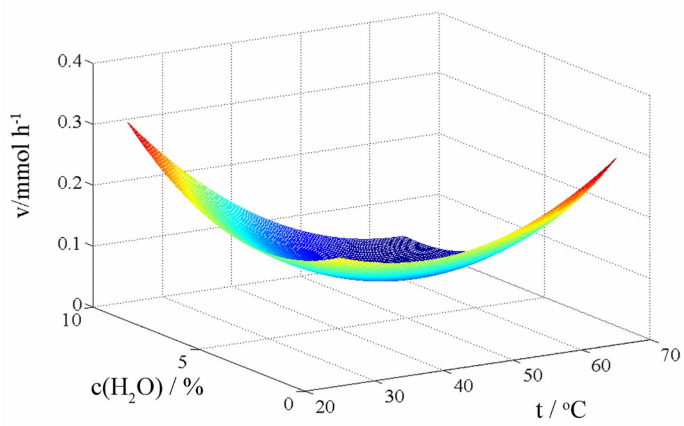


Fig. 2. The response surface of the reaction rate per unit amount of enzyme at different levels of water amount and temperature.

Comparing the two obtained models, it is clear that in the first model the amount of enzyme is the most significant factor with positive effect on the production rate of biodiesel, whereas in the second model it did not influence the production rate of biodiesel per unit amount of enzyme. These findings indicate that, although with increasing amount of enzyme in the system the rate of biodiesel production was increased, it is not economically justified. However, reports can be found that increasing the enzyme concentration up to 30 % led to an increase in the yield of biodiesel.[7] This implies that the rate of transesterification per unit amount of enzyme should be the response variable of choice in the development of the best method for evaluation of the effectiveness of a production process of biodiesel.

In both models, the methanol/oil molar ratio (3:1–9:1) was not a significant factor for the synthesis of biodiesel. In a majority of related studies, the methanol/oil molar ratio significantly influenced the yield of methyl esters. For example, Soumanou *et al.* also investigated the influence of methanol on the yield of biodiesel, with the same lipase, and obtained best results with 3:1 methanol/oil molar ratio.[8] In this case, the enzyme concentration was significantly higher (10 %), and even a slight increase of excess methanol resulted in the almost complete inactivation of the lipase. In a different study, the best results were obtained at 4:1 alcohol/oil molar ratio with an even higher amount of enzyme (27 %). However, the activity of the immobilized enzyme from *Candida antarctica* decreased at higher methanol concentrations (5:1 and 6:1).[10]

The addition of water exhibited a negative effect on the reaction rate. The highest activity was achieved without addition of water to the reaction mixture (Figs. 1b and 1c). Increasing the amount of added water up to 6.5 % led to a steep decrease of activity but further increase of the amount of water led to a slight increase of the transesterification activity. Such a behavior is a consequence of the complex reaction mechanism of enzymatic transesterification.[22] The amount of water in the system should be a compromise between minimizing hydrolysis and maximizing enzyme activity for the transesterification reaction.[11] Immobilized enzymes, however, show the highest activity in low-water systems. The highest initial rate of the transesterification reaction using immobilized *Psendomonas fluorescens* was achieved at 0.3 % of added water.[23] The capability of lipase from *R. miehei* to catalyze reactions at exceptionally low activity of water, a_{W}, was documented in a previous study.[24] It was reported that the enzyme remained highly active at a water activity below 0.0001.

In this study, according to the test of statistical significance, only one interactive effect was observed, *i.e.*, a negative interactive effect between the temperature and water content on the reaction rate per unit amount of enzyme. It can be seen from the response surface (Fig. 2) that a higher reaction rate could be obtained at a high temperature and low water content, or *vice versa*. The transesterification activity of lipase *R. miehei* rapidly decreased with the combined increase of both the amount of water and temperature, probably due to lipase denaturation. Additionally, with excess water, the hydrolysis of methyl esters is favorable, especially at high temperatures.

CONCLUSIONS

The response surface methodology proved to be a valuable tool for evaluating the effects of various factors on the lipase-catalyzed synthesis of biodiesel fuel from sunflower oil. The reaction temperature and the amount of added water significantly influenced the rate of biodiesel production and these effects were adequately described by a quadratic model. Most importantly, RSM enabled the determination of a negative interactive effect between temperature and the amount of water, which would have been unseen in a classical investigation of the effects by variation of one factor while keeping the other parameters at fixed values. Thus, the highest yield of biodiesel was achieved at 45 °C and without water addition. Additionally, the results of this research imply that the choice of output parameter is of crucial importance, since different models were obtained with two particular response variables. The enzyme concentration had the most pronounced effect on the rate of biodiesel production but had no effect on the rate of biodiesel production per unit amount of enzyme. This fact implies that enzymatic synthesis of biodiesel with high amounts of lipase is not economically acceptable in a batch process, in spite of a certain increase of the product yield.

ИЗВОД

ОПТИМИЗОВАЊЕ ПОСТУПКА ПРОИЗВОДЊЕ БИОДИЗЕЛА КОМЕРЦИЈАЛНОМ ЛИПАЗОМ У СИСТЕМУ БЕЗ ОРГАНСКОГ РАСТВАРАЧА ПРИМЕНОМ МЕТОДОЛОГИЈЕ ОДЗИВНИХ ПОВРШИНА

НЕВЕНА ОГЊАНОВИЋ, ДЕЈАН БЕЗБРАДИЦА и ЗОРИЦА КНЕЖЕВИЋ

Технолошко-металуршки факултет, Карнегијева 4, 11000 Београд

Циљ овог рада била је оптимизација процеса синтезе биодизела коришћењем имобилисане липазе из *Rhizomucor miehei*. У овом раду испитан је утицај основних процесних параметара на принос биодизела: температуре, моларног односа метанол/уље, концентрације ензима и концентрације воде у систему без органског растварача. Оптимизација процеса ензимске синтезе је извршена коришћењем ротатабилног композиционог плана. За обраду података коришћена је методологија одзивних површина. Установљено је да се утицај фактора на брзину ензимске реакције може описати моделом другог реда са три фактора, без међусобне интеракције. Статистичка анализа показује да је најзначајнији параметар количина ензима у реакционој смеши. Повишена температура је имала негативан утицај на брзину ензимске реакције, као и повећана концентрација воде. За анализу утицаја различитих фактора на брзину ензимске реакције по количини ензима, добијен је модел другог реда, са два значајна параметра са међусобном интеракцијом. Из модела се види да је модел изузетно стабилан при малом садржају воде, а да реакцији погодује велика количина воде само при ниским температурама. Највећи принос, од 10,15 mol kg^{-1} ензима, остварен је при следећим параметрима реакције: 45 °C, моларни однос метанол/уље 6:1 и без додате воде у систем.

(Примљено 14. марта, ревидирано 2. јула 2007)

REFERENCES

1. J. M. Encinar, J. F. Gonzales, J. J. Rodriguez, A. Tejedor, *Energy Fuels* **16** (2002) 443
2. A. W. Schwab, M. O. Bagby, B. Freedman, *Fuel* **66** (1987) 1372
3. S. Saka, D. Kusdiana, *Fuel* **80** (2001) 225
4. Y. Shimada, Y. Watanabe, A. Sugihara, Y. Tominaga, *J. Mol. Catal. B: Enzym.* **17** (2002) 133
5. D. Kusdiana, S. Saka, *Fuel* **80** (2001) 693
6. D. Wei, X. Yuanyan, L. Dehua, J. Zeng, *J. Mol. Catal. B: Enzym.* **30** (2004) 125
7. O. Kose, M. Tuter, H. A. Aksoy, *Bioresour. Technol.* **83** (2002) 125
8. M. M. Soumanou, U. T. Bornscheuer, *Enzyme Microb. Technol.* **33** (2003) 97
9. M. Kaieda, T. Samukawa, A. Kondo, H. Fukuda, *J. Biosci. Bioeng.* **91** (2001) 12
10. M. Kaieda, T. Samukawa, T. Matsumoto, K. Ban, A. Kondo, Y. Shimada, H. Noda, K. Ohtsuka, E. Izumoto, H. Fukuda, *J. Biosci. Bioeng.* **88** (1999) 627
11. H. Noureddini, X. Gao, R. S. Philkana, *Bioresour. Technol.* **96** (2005) 769
12. C. G. Laudani, M. Habulin, M. Primozic, Z. Knez, G. Della Porta, E. Reverchon, *Bioprocess Biosyst. Eng.* **29** (2006) 119
13. F. J. Hernandez, A. P. de los Rios, D. Gomez, M. Rubio, G. Villora, *Appl. Catal. B* **67** (2006) 121
14. L. A. Nelson, T. A. Foglia, W. N. Marmer, *J. Am. Oil Chem. Soc.* **73** (1996) 1191
15. A. Salis, M. Pinna, M. Monduzzi, V. Solinas, *J. Biotechnol.* **119** (2005) 291
16. G. Vicente, A. Coteron, M. Martimez, J. Aracil, *Ind. Crops Prod.* **8** (1998) 29
17. C. J. Shieh, H. F. Liao, C. C. Lee, *Bioresour. Technol.* **88** (2003) 103

18. Z. Knežević, N. Ognjanović, D. Bezbradica, in *Proceedings of the 1st International Symposium on Environmental Biocatalysis*, Cordoba, Spain, 2006, p. 56
19. 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
20. Z. Knežević, N. Milosavić, D. Bezbradica, Ž. Jakovljević, R. Prodanović, *Biochem. Eng. J.* **30** (2006) 269
21. B. Manohar, S. Divakar, *Process Biochem.* **39** (2004) 847
22. H. Fukuda, A. Kondo, H. Noda, *J. Biosci. Bioeng.* **92** (2001) 405
23. M. Iso, B. Chen, M. Eguchi, T. Kudo, S. Shrestha, *J. Mol. Catal. B: Enzym.* **16** (2001) 53
24. R. H. Valivety, P. J. Halling, A. R. Macrae, *FEBS Lett.* **301** (1992) 258.