Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 45 (2007) 97–101
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

The effect of substrate polarity on the lipase-catalyzed synthesis of aroma esters in solvent-free systems

Dejan Bezbradica [a,*], Dušan Mijin [b], Slavica Šiler-Marinković [a], Zorica Knežević [a]

[a] *Department for Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, Karnegijeva 4, Belgrade, Serbia*
[b] *Department for Organic Chemistry, Faculty of Technology and Metallurgy, Karnegijeva 4, Belgrade, Serbia*

Received 15 May 2006; received in revised form 5 December 2006; accepted 18 December 2006
Available online 23 December 2006

Abstract

The aim of this study was to compare activities of commercial lipases in synthesis of various esters in solvent-free system and in isooctane. Moreover, the effect of substrate polarity (expressed as log *P*) on solvent-free synthesis was investigated. The decrease of yields of esters of butanoic acid in absence of organic solvent was observed, while similarly high yields were noticed in synthesis of esters of octanoic acid in both systems (solvent-free and organic solvent). The kinetic analysis has shown that ester synthesis can be described with Ping-pong bi-bi kinetics. In a case of esterification of butanoic acid in solvent-free system additional term, which represents enzyme inactivation by acid substrate, must be included. It was found out that log *P* of initial substrate mixture was in linear correlation with kcat of ester synthesis, while final yields depend only on type of acid substrate. Each of the examined lipases showed similar properties, although immobilized lipase from *Rhizomucor miehei* was slightly more resistant to harmful influence of butanoic acid. Finally, it was also shown that detrimental influence of butanoic acid could be circumvented by two-step addition of acid substrate in reaction catalyzed with immobilized lipase from *R. miehei*.

Keywords: Lipase; Aroma esters; Log *P*; Solvent-free synthesis; *Rhizomucor miehei*

1. Introduction

Aroma esters are important ingredients of variety of products of food industry. Although these important products are manufactured mostly by a chemical method that includes use of aggressive chemical catalysts, development of the research area of enzymatic esterification is very propulsive during previous two decades [1]. An important impetus for development of enzymatic synthesis of flavor and fragrance esters was the fact that esters produced in such a way can be labeled as natural [2]. Numerous reports of achieving high yields of esters with various lipases (triacylglycerol hydrolases) of microbial origin have been published [3–5]. The enzymatic ester synthesis was prevalently performed in various organic media with low water content because lipase has stable active conformation in these systems and improved thermostability [6]. The downsides are high price, toxicity and flammability of organic solvents and higher investment costs in order to meet safety requirements. The enzymatic processes in solvent-free systems are attractive for the synthesis of esters with the lower melting points because of considerable simplification of the downstream processing and reduced environmental hazard. Additional advantages of reactions in solvent-free system are savings in reactor design in large-scale process and reduction of separation costs due to avoiding solvent recovery [7,8]. Therefore, studies of the solvent-free ester synthesis are more frequent during last decade. Ghamgui et al. obtained high yields of buthyl oleate by using lipase from *Rhizopus oryzae* immobilized on $CaCO_3$ [9], while Garcia et al. successfully performed synthesis of isopropyl palmitate with highly active lipase from *Candida antarctica* [10]. Significant activity in solvent-free synthesis of ethyl oleate was observed for lipase from *Rhizomucor miehei*, free and immobilized on polyamide support [11]. Isoamyl acetate (banana flavor) was obtained with yields over 60% by immobilized lipase from *Staphylococcus simulans* [12]. Goldberg et al. performed syntheses of heptyl octanoate and heptyl oleate by lipase from *Candida cylindracea* in solvent-free system and achieved yields over 50% [8]. Additionally,

* Corresponding author. Fax: +381 11 3370 387.
E-mail address: dbez@tmf.bg.ac.yu (D. Bezbradica).

1381-1177/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.12.003

solvent-free lipase-catalyzed systems has been used recently for the synthesis of long-chain acyl thioesters [13], amidation of carboxylic acids [14], transesterification of high-oleic sunflower oil [15] and synthesis of esters of docosahexaneoic acid [16].

In some of above-mentioned studies, solvent-free esterification was compared with esterification in organic solvent, but the influence of physicochemical properties of alcohol or acid substrate on efficiency of solvent-free synthesis was not thoroughly investigated. In this study syntheses of esters of various lengths were performed with porcine pancreatic lipase. Parallel experiments in solvent-free system and in isooctane were performed, and the influence of substrate polarity (expressed as log *P*) on lipase-activity in solvent-free system was studied.

2. Experimental

2.1. Materials

Porcine pancreatic lipase (triacylglycerol hydrolase, EC 3.1.1.3) was provided by Sigma (St. Louis, USA). Lipozyme (immobilized lipase from *R. miehei*) was kindly gifted by Novo Nordisk (Bagsvaerd, Denmark). Isooctane was provided by Fluka (Buchs, Switzerland). Butanoic acid and *n*-pentanol were provided by Farmitaliana Carlo Erba (Milano, Italy). Hexanoic acid, octanoic acid, *n*-heptanol, *n*-octanol, and *n*-propanol were purchased from Fluka (Buchs, Switzerland). Geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol) was purchased from Acros Organics (New Jersey, USA). All chemicals were of 99% or higher purity. Hydranal® solvent and Hydranal® titrant 5 was purchased from Riedel de Haen (Seelze, Germany).

2.2. Methods

Specific activities were 141 and 157 IU/g for pancreatic lipase and for lipozyme, respectively, determined by Sigma method [17], where 1 IU is defined as the amount of enzyme required to produce 1 μmol of free fatty acid per min under the assay conditions (37 °C, pH 7.7). Ester synthesis was carried out in stoppered flasks (100 ml) in isooctane (up to 10 ml of reaction mixture) or solvent-free system. The reaction mixture, containing 50 mg of enzyme, 10 μl of water and 2.5×10^{-3} mol of each substrate, was incubated on a shaker at 150 rpm and at 45 °C. Two-step addition of acid substrate was carried out with addition of first portion of acid (1.25×10^{-3} mol) in the initial reaction mixture, and addition of second portion (1.25×10^{-3} mol) in the moment when almost entire amount of acid was converted to ester. Each experimental data was obtained by performing the synthesis in separate flasks. All of the experiments were carried out in duplicate. Average values and standard deviations of experimental results are presented in figures. Control experiments (without enzyme) were performed and it was observed that ester yield is negligible (below 2%).

The progress of esterification was monitored by determination of the residual acid content by titration against sodium hydroxide using phenolphthalein as an indicator and mixture of ethanol and diethyl ether (1:1) as a quenching agent. The amount of ester was calculated as being equivalent to acid consumed. The accuracy of this method was tested by determination of ester concentration on Varian 3400 gas chromatograph equipped with a DB-1 capillary column and a flame ionization detector. Nitrogen was used as a carrier gas. The initial reaction rates were determined from the slope of the initial linear portions of the plots of ester concentration versus time. Initial rates and yields determined by GC analysis and titrimetry were in good agreement.

The kinetic analysis of concentration–time curves was performed by using software Encora version 1.2 [18].

Equilibration of reaction mixtures at $a_w = 0.67$ was performed in desiccators above a saturated solution of $CuCl_2 \cdot 2H_2O$. The equilibration was performed for 2 days at room temperature.

The measurement of water concentration was performed on the Karl–Fischer apparatus (Mettler Toledo, USA). Water concentrations in solvent-free reactions were in narrow range between 1.52 and 1.69%.

3. Results and discussion

A comparative study of pancreatic lipase-catalyzed esterification of *n*-butanoic acid in solvent-free system and in isooctane was carried out with three alcohols: *n*-pentanol, *n*-heptanol and geraniol. Obtained results are illustrated in Fig. 1. In solvent-free system final yield was reached significantly faster (within first 15 h) than in reactions carried out in isooctane, but it is evident that obtained yields were below 20%, while the final yields in isooctane were around 90%. The results of kinetic analysis (performed with Encora 1.2 software) imply that different kinetic models describe ester synthesis in solvent-free system and in organic solvent. The synthesis in organic solvent can be described with basic Ping-pong bi-bi kinetic model (Eq. (1)), while for solvent-free synthesis enzyme inactivation by acid sub-

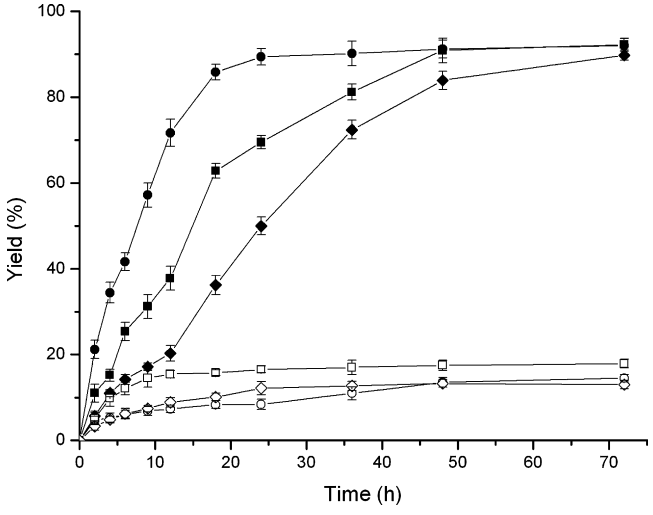

Fig. 1. The synthesis of esters of butanoic acid with porcine pancreatic lipase. Reaction conditions: $t = 45$ °C, $n_0(\text{Al}) = n_0(\text{Ac}) = 2.5 \times 10^{-3}$ mol; $V_0(H_2O) = 10$ μl; m(E) = 50 mg. (■) Pentyl butanoate (in isooctane); (●) heptyl butanoate (in isooctane); (♦) geranyl butanoate (in isooctane); (□) pentyl butanoate (solvent-free); (○) heptyl butanoate (solvent-free); (◊) geranyl butanoate (solvent-free). When reactions were performed in organic solvent, volume of reaction mixture was 10 ml.

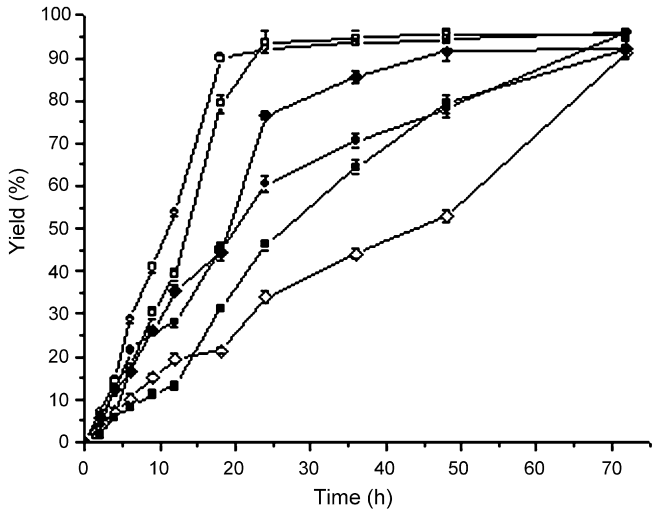

Fig. 2. The synthesis of esters of octanoic acid using porcine pancreatic lipase. Reaction conditions: $t = 45\,^{\circ}C$, $n_0(Al) = n_0(Ac) = 2.5 \times 10^{-3}$ mol; $V_0(H_2O) = 10\,\mu l$; m(E) = 50 mg. (■) Octyl octanoate (in isooctane); (●) heptyl octanoate (in isooctane); (♦) propyl octanoate (in isooctane); (□) octyl octanoate (solvent-free); (○) heptyl octanoate (solvent-free); (◊) propyl octanoate (solvent-free). When reactions were performed in organic solvent, volume of reaction mixture was 10 ml.

strate must be included in Ping-pong bi-bi kinetic model (Eq. (2)):

$$v = \frac{V_{max}[Al][Ac]}{[Al][Ac] + K_{Al}[Ac] + K_{Ac}[Al]} \quad (1)$$

$$-\frac{d[E]}{dt} = k_D[E][Ac] \quad (2)$$

where v is the initial reaction rate, V_{max} the maximum reaction rate, K_{Al} and K_{Ac} Michaelis constants of alcohol and acid, and k_D is the constant of enzyme deactivation. The results were in a good agreement with kinetic models—divergences of model were less than 10% in all experiments. The obtained rate constants are illustrated in Table 1. It can be seen (Table 1) that k_{cat} is 5–6 times higher in an organic solvent than in the solvent-free synthesis.

In Fig. 2 reaction curves of pancreatic lipase-catalyzed synthesis of esters of *n*-octanoic acid (with *n*-propanol, *n*-heptanol and *n*-octanol) in a solvent-free system and in isooctane were presented. In this experimental series pancreatic lipase showed somewhat different behavior than during synthesis of esters of *n*-butanoic acid. High final yields of ester (each above 85%) were achieved in all experiments. Opposite to previous part of the study, the final yields were higher in a solvent-free system, although differences were less significant. The kinetic analysis has shown that reaction in both systems could be described by Ping pong bi-bi kinetics (Table 1). Additionally, initial rates were very high in solvent-free system. The solvent-free system was especially advantageous for the synthesis of heptyl octanoate and octyl octanoate because yields above 90% were reached within 20 h, while in reaction performed in isooctane similar yields were achieved after 70 h. After comparison of kinetic parameters of synthesis of esters of butanoic and octanoic acid (Table 1), it can be concluded that if octanoic acid is used as acyl donor, k_{cat} is significantly higher in solvent-free system, while in synthesis in organic solvent no significant difference between various acyl donors has been noticed. In addition, it can be noticed that the type of applied acid substrate was even more important for achieving high yields of esters in solvent-free system. Esters of butanoic acid could not be obtained with yields higher than 20% in solvent-free system, while significantly higher yields were achieved for esters of octanoic acid. It is plausible that such behavior of pancreatic lipase is consequence of higher polarity of butanoic acid in comparison with octanoic acid. It is well known fact that certain water micro-layer around enzyme molecule is necessity for keeping the enzyme in its active conformation and that polar substances readily destroy this layer [19,20]. That is the most important reason for application of non-polar organic compounds as solvents for enzymatic synthesis. Lower yields in solvent-free system could be a consequence of damaging water layer by high concentration of polar substrate in a vicinity of enzyme. Additionally, some researchers emphasized that enhanced dissociation of weak organic acid leads to the increase of rate of the reverse reaction (hydrolysis) [20]. This could be another explanation of low yields in synthesis of esters of butanoic acid, since dissociation constant of butanoic acid is lower in comparison with octanoic acid (1.52×10^{-5} and 1.29×10^{-5}, respectively). It must be emphasized that propanol,

Table 1
The kinetic constants of ester synthesis catalyzed with porcine pancreatic lipase

Ester	$K_{m,Ac}$ (M)	$K_{m,Al}$ (M)	k_{cat} (mmol h^{-1} g^{-1})	K_d (h^{-1} M^{-1})
Heptyl butanoate	0.6120	7.0180	18.000	–
Heptyl butanoate (s.f.)	0.6242	7.0640	2.8940	0.058
Geranyl butanoate	0.60264	6.95634	7.132	–
Geranyl butanoate (s.f.)	0.66778	6.93217	1.428	0.064
Pentyl butanoate	0.6034	6.9885	11.114	–
Pentyl butanoate (s.f)	0.6100	6.9948	2.222	0.082
Propyl octanoate	0.6184	6.9594	8.0872	–
Propyl octanoate (s.f.)	0.63665	6.913	3.6715	–
Heptyl octanoate	0.65010	8.47448	7.41308	–
Heptyl octanoate (s.f.)	0.623	7.756	9.85	–
Octyl octanoate	0.6234	6.9177	4.821	–
Octyl octanoate (s.f.)	0.6211	6.8974	11.104	–
Heptyl hexanoate	0.6161	6.9968	11.22	–
Heptyl hexanoate (s.f.)	0.624	6.9265	4.856	–

which was also used as substrate, is even more polar than butyric acid but enzyme inactivation was not observed in its presence. Moreover, polar acid substrate is probably more harmful for vicinity of active site of enzyme due to the fact that it was postulated in numerous studies that prerequisite for enzymatic esterification is formation of acyl-enzyme complex [21].

In order to elucidate the role of water in discrepancies between yields in different systems additional experimental series was performed. In these experiments both systems were saturated to same water activity ($a_w = 0.67$) prior to esterification. It seems water activity did not have significant influence on the esterification since yield of ester in solvent-free reaction was still significantly lower (27%, compared with 94% in isooctane).

On the other hand, in the synthesis of heptyl octanoate and octyl octanoate considerably higher initial rates of ester formation were observed in solvent-free system. It is plausible that in system that contains only medium-chain substrates there is no occurrence of destruction of water layer, which was limiting phenomenon in the synthesis of esters of butanoic acid. Therefore, higher initial rate of solvent-free synthesis could be consequence of higher concentration of substrates in the vicinity of enzyme, which led to higher number of efficient enzyme-substrate collisions.

The next part of this study was focused on finding correlation between change in lipase activity in solvent-free system (compared with reaction in organic solvent) and properties of substrate mixture. Several physicochemical properties of reaction media have been tested during previous few decades for prediction of biocatalytic activity [22]. The logarithm of the octanol/water partition coefficient for solvent (log P) is usually used as parameter that guide the choice of solvent in lipase-catalyzed reactions, because it was determined in a number of studies that activity of lipases increased with an increase of log P [23]. Therefore, in this study was investigated if discrepancies between lipase activities in solvent-free system and organic solvent could be correlated with log P value of reaction mixture. Log P values were calculated by Eq. (3):

$$\log P = x_1(\log P)_1 + x_2(\log P)_2 \quad (3)$$

where x_1 and x_2 represent molar fractions of substrates, while $(\log P)_1$ and $(\log P)_2$ stand for log P values of substrates. Literature values for log P of substrates [24] and calculated values for substrate mixtures are shown in Table 2. The attempt was made to find correlation between ratios of k_{cat} in different reaction systems and log P value of reaction mixtures. As it can be seen from Fig. 3 strong linear correlation between logarithm of k_{cat} ratios and log P exists at log P values above 1.75 (linearity coefficient 0.987). On the other hand, at log P values below 1.75, k_{cat} ratio became almost constant at value that indicates favorable synthesis in organic solvent. It can be seen (Fig. 4) that there was no quantitative correlation between the final yield of ester and initial log P of substrate mixture, but it seems that type of acid substrate has crucial influence on final yield. Specifically, yields of esters of butanoic acid were drastically lower in solvent-free system compared with yields of esters of hexanoic or octanoic acid.

Table 2
Literature values of log P of individual substrates and calculated values for initial substrate mixtures

Acid	Log P	Alcohol	Log P	Log P of substrate mixture
n-Butanoic	0.79	*n*-Pentanol	1.51	1.18
		n-Heptanol	2.62	1.90
		Geraniol	3.56	2.61
n-Octanoic	3.05	*n*-Propanol	0.25	2.58
		n-Heptanol	2.62	2.85
		n-Octanol	3.00	3.03

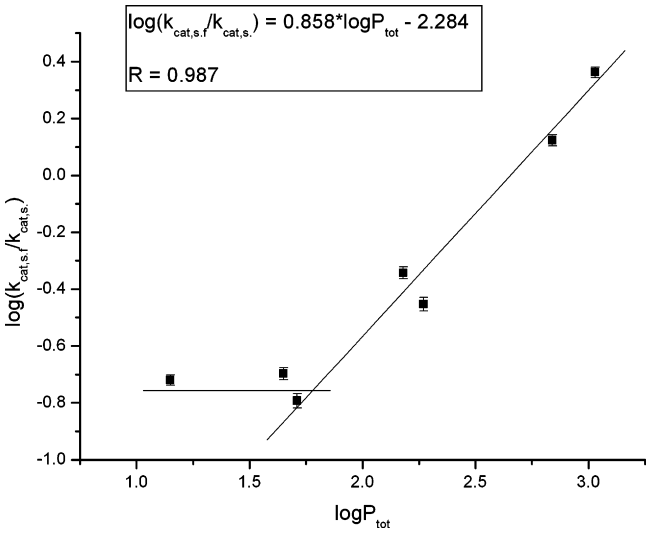

Fig. 3. The effect of log P of initial substrate mixture on the initial rate of the ester synthesis.

The results of previously mentioned experiments showed that medium-chain esters could be obtained in lipase-catalyzed synthesis with yields higher than 90% in solvent-free system. The attempt was made to overcome the problem of low yields of esters of butyric acid by two-step addition of butanoic acid

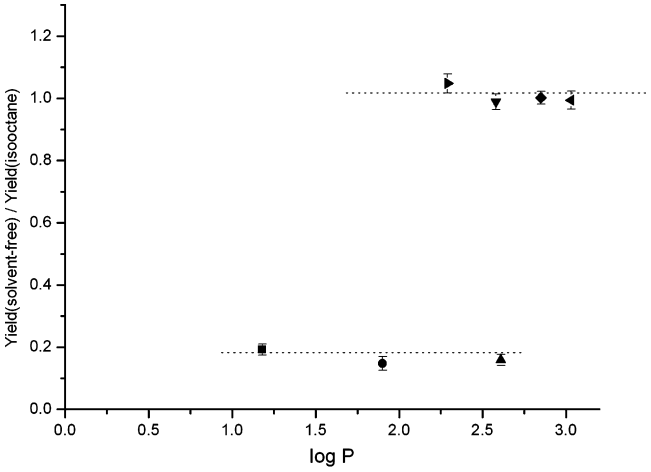

Fig. 4. The effect of log P of initial substrate mixture on the final yield of the ester synthesis. (◀) Octyl octanoate; (▼) propyl octanoate; (►) heptyl hexanoate; (▲) geranyl butanoate; (♦) heptyl octanoate; (■) pentyl butanoate; (●) heptyl butanoate.

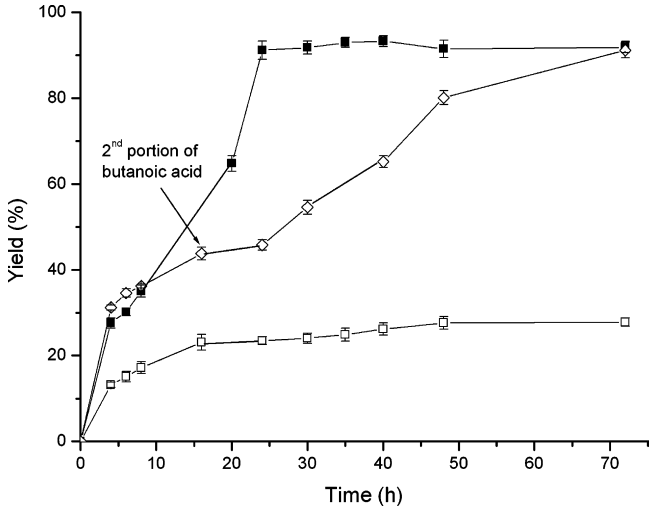

Fig. 5. The synthesis of pentyl butanoate using two-step addition of acid substrate with immobilized lipase from *R. miehei*. Reaction conditions: $t = 45\,^{\circ}C$, $n_0(Al) = 2.5 \times 10^{-3}$ mol; $n_0(Ac) = 1.25 \times 10^{-3}$ mol; $V_0(H_2O) = 10\,\mu l$; m(E) = 50 mg. When reactions were performed in organic solvent the volume of reaction mixture was 10 ml. (■) One-step process (in isooctane); (□) one-step process (solvent-free); (◊) two-step process (solvent-free).

in reaction mixture. This approach was successful in reducing inhibitory effect of methanol in lipase-catalyzed synthesis of biodiesel [25]. The synthesis of pentyl butanoate was used as a model system. There was no improvement in comparison with one-step esterification with pancreatic lipase (results not shown). Nevertheless, results (depicted in Fig. 5) showed that high yield of ester (similar to reaction in isooctane) was obtained by performing esterification in such a way when immobilized lipase from *R. miehei* was used. It is plausible that irreversible change of conformation of pancreatic lipase occurs due to destruction of water layer around enzyme molecule, while immobilized lipase was more resistant because a certain amount of water is retained in support.

4. Conclusion

The synthesis of various aliphatic esters was successfully performed with porcine pancreatic lipase and immobilized lipase from *R. miehei*. The obtained results indicate that solvent-free reactions have great potential for enzymatic synthesis of medium-chain esters with different enzyme preparations. In this study was also shown that discrepancies between kinetic properties of lipase in different reaction media (solvent-free and organic solvent media) can be correlated with log *P* values of substrate mixtures. Consequently, the prediction of efficiency of lipase in solvent-free synthesis is feasible if log *P* values of both substrates are known. The main obstacle to successful application of solvent-free synthesis was the increased susceptibility of enzymes toward polar acid substrates and their faster denaturation. This drawback was successfully circumvented by using two-step addition of acid substrate in synthesis catalyzed by immobilized lipase from *R. miehei*. It may be concluded that various aroma esters can be successfully produced in a solvent-free system using inexpensive free or immobilized lipases in an environmental-friendly conditions.

References

[1] R.M. Yahia, W.A. Anderson, M. Moo-Young, Enzyme Microb. Technol. 23 (1998) 438.
[2] H. Larios, S. Garcia, R.M. Oliart, G. Valerio-Alfaro, Appl. Microbiol. Biotechnol. 65 (2004) 373.
[3] S. Hari Krishna, B. Manohar, S. Divakar, S.G. Prapulla, N.G. Karanth, Enzyme Microb. Technol. 26 (2000) 131.
[4] G.V. Chowdary, M.N. Ramesh, S.G. Prapulla, Process Biochem. 36 (2000) 331.
[5] X.L. He, B.Q. Chen, T.W. Tan, J. Mol. Catal. B: Enzym. 18 (2002) 333.
[6] A. Zaks, A.M. Klibanov, Science 224 (1984) 1249.
[7] S. Bloomer, P. Adlercreutz, B. Mattiasson, Enzyme Microb. Technol. 14 (1992) 546.
[8] M. Goldberg, F. Parvaresh, D. Thomas, M.-D. Legoy, Enzyme Microb. Technol. 12 (1990) 976.
[9] H. Ghamgui, M. Karra-Chaabouni, Y. Gargouri, Enzyme Microb. Technol. 35 (2004) 355.
[10] T. Garcia, N. Sanchez, M. Martinez, J. Aracil, Enzyme Microb. Technol. 25 (1999) 584.
[11] A.C. Oliveira, M.F. Rosa, M.R. Aires-Barros, J.M.S. Cabral, J. Mol. Catal. B: Enzym. 11 (2001) 999–1005.
[12] H. Ghamgui, M. Karra-Chaabouni, S. Bezzine, N. Miled, Y. Gargouri, Enzyme Microb. Technol. 38 (2006) 788.
[13] N. Weber, E. Klein, K.D. Mukherjee, Appl. Microbiol. Biotechnol. 51 (1999) 401.
[14] A.K. Prasad, M. Husain, B.K. Singh, R.K. Gupta, V.K. Manchanda, C.E. Olsen, V.S. Parmar, Tetrahedron Lett. 46 (2005) 4511.
[15] V. Dossat, D. Combes, A. Marty, J. Biotechnol. 97 (2002) 117.
[16] Y. Shimada, Y. Watanabe, A. Sugihara, T. Baba, T. Ooguri, S. Moriyama, T. Terai, Y. Tominaga, J. Biosci. Bioeng. 92 (2001) 19.
[17] Z. Knezevic, N. Milosavic, D. Bezbradica, Z. Jakovljevic, R. Prodanovic, Biochem. Eng. J. 30 (2006) 269.
[18] A.J.J. Straathof, J. Mol. Catal. B: Enzym. 11 (2001) 991.
[19] D.T. Lai, C.J. O'Connor, J. Mol. Catal. B: Enzym. 6 (1999) 411.
[20] S. Hari Krishna, S. Divakar, S.G. Prapulla, N.G. Karanth, J. Biotechnol. 87 (2001) 193.
[21] X. Malcata, H. Reyes, H. Garcia, C. Hill, C. Amundson, Enzyme Microb. Technol. 14 (1992) 426.
[22] D. Nurok, R. Kleyle, B. Muhoberac, M. Frost, P. Hajdu, D. Robertson, S. Kamat, A. Russell, J. Mol. Catal. B: Enzym. 7 (1999) 273.
[23] M.H. Vermue, J. Tramper, Pure Appl. Chem. 67 (1995) 345.
[24] http://esc.syrres.com/interkow/webprop.exe.
[25] Y. Watanabe, Y. Shimada, A. Sugihara, Y. Tominaga, J. Mol. Catal. B: Enzym. 17 (2002) 151.