Journal of Molecular Catalysis B: Enzymatic 71 (2011) 85–89

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Improved esterification activity of *Candida rugosa* lipase in organic solvent by immobilization as Cross-linked enzyme aggregates (CLEAs)

Funda Kartal[a,*], Michiel H.A. Janssen[b], Frank Hollmann[c], Roger A. Sheldon[b,c], Ali Kılınc[a]

[a] *Biochemistry Department, Science of Faculty, Ege University, 35100 Bornova/Izmir, Turkey*
[b] *CLEA Technologies BV, Julianalaan 136, 2628 BL Delft, The Netherlands*
[c] *Laboratory of Biocatalysis and Organic Chemistry, Delft University of Technology, Julianalaan 136, 2628 BL Delft, The Netherlands*

ARTICLE INFO

Article history:
Received 29 December 2010
Received in revised form 25 March 2011
Accepted 5 April 2011
Available online 14 April 2011

Keywords:
Lipase
Immobilization
Cross-linked enzyme aggregates
Esterification
Surfactant

ABSTRACT

Cross-linked enzyme aggregates (CLEA®s) were prepared from *Candida rugosa* lipase (CrL) using glutaraldehyde as the cross-linker. The optimum conditions of the immobilization process were determined (precipitant: ethanol, crosslinker concentration: 25 mM, enzyme concentration: 50 mg/ml, crosslinking time: 45 min.). CLEAs were shown to have several advantages compared to the free enzyme. They were more stable at 50 °C and 60 °C and had good reusability; retaining 40% of their initial activity after 15 recycles in aqueous media and remaining constant at that level thereafter, suggesting some initial leaching in water. The CLEAs catalyzed esterification reactions in cyclohexane, affording higher conversions than with the free enzyme, especially when longer fatty acids and alcohols were used as substrates.

1. Introduction

Enzymes are useful tools for performing industrially important chemical reactions in a stereo-, regio- and chemoselective manner. In many cases, e.g. in the production of fine chemicals, pharmaceuticals, agrochemicals, fragrances and flavors, food additives and personal care products, industrial viability requires that enzymes are stable for extended periods in organic solvents. This can often be achieved by immobilization which also improves the economics of the process by facilitating recovery and reuse of the biocatalyst. However, immobilization is often costly and generally requires an inert matrix on which the enzyme can be immobilized. Moreover, the matrix must often be chemically modified to enable covalent coupling of the enzyme. Immobilization of an enzyme on an inert support dilutes the effective concentration of the enzymes. In contrast, cross-linking of enzymes results in both stabilization and immobilization without dilution of activity [1].

Cross-linked enzyme aggregates (CLEAs) have been proposed as an alternative to conventional immobilization on pre-existing solid supports [2]. The preparation of CLEAs involves the precipitation of the enzyme (that need not be pure) and subsequent chemical cross-linking of the protein aggregates. Physical aggregation of enzyme molecules into supermolecular structures can be induced, without perturbation of the original three-dimensional structure of the protein, by the addition of salts, organic solvents or non-ionic polymers to an aqueous solution of the protein. These solid aggregates are held together by noncovalent bonding and readily collapse and redissolve when dispersed in an aqueous medium. It has been suggested [3] that the chemical cross-linking of these physical aggregates produces cross-linked enzyme aggregates in which the reorganized superstructure of the aggregates and, hence, their activity is maintained. The cross-linking step is necessary to stabilize the aggregates so that they cannot be redissolved when removing the precipitating reagent. The preparation of CLEAs of a wide variety of enzymes [3] including various lipases [4–14] has been described.

Lipases are widely used in organic synthesis and, hence, there is much interest in effective methods for their immobilization [4]. The use of lipases in esterification reactions to produce industrially important products such as emulsifiers, surfactants, wax esters, biopolymers, modified fats and oils, structured lipids and flavor esters is well documented [15]. Esters of short-chain alcohols and short chain fatty acids are important aroma compounds [16]. Esters of short chain alcohols and long-chain fatty acids are valuable oleochemicals that may be used as lubricants, diesel fuel and antistatic reagents [17]. Esters of long chain fatty acids and polyhydric alcohols like glycerol, sorbitol and other carbohydrates find numerous applications in the food and pharmaceutical industries [18].

Candida rugosa (formerly *Candida cylindracea*) lipase (CrL) is widely applied in biotransformations of fats and oils [19], e.g. in the synthesis of wax esters [20]. It is actually a mixture of several isoforms, the exact composition of which is dependent on the sup-

* Corresponding author. Tel.: +90 232 311 24 55; fax: +90 232 343 86 24.
E-mail address: funda.kartal@ege.edu.tr (F. Kartal).

1381-1177/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2011.04.002

Table 1
Precipitant effect on activities of precipitated enzyme and CLEAs.

Precipitant type	CLEA presence	(%) Activity recoveries after precipitation	(%) Activity recoveries after crosslinking
Ammonium sulfate (saturated sol.)	+	18	0.5
Ethanol	+	71	8.5
Tert-butanol	–	78	–
Acetone	+	80	no-activity
Iso-propanol	+	94	0.9

Conditions: precipitation step: precipitant:enzyme soln. (v/v) 9:1, crosslinking step: crosslinking time: 3h, [glutaraldehyde]: 25 mM, and [enzyme]: 50 mg/ml.

plier [19]. Scant attention has been devoted to the preparation of CLEAs from CrL [5,21,22] and their possible benefits in practical applications. Herein we report the preparation of *C. rugosa* lipase CLEAs and a comparison of their thermostability, reusability and tolerance to organic solvents, in industrially relevant esterification reactions, with those of the free enzyme.

2. Materials and methods

2.1. Chemicals

Lipase from *C. rugosa* (Lipomod^{TM} 34P) was kindly donated by Biocatalysts (Cardiff, United Kingdom), glutaraldehyde (25%, w/v solution) was from Sigma–Aldrich (Zwijndrecht, the Netherlands). All other chemicals were analytical grade.

2.2. General CLEA preperation

Cross-linked aggregates of *C. rugosa* lipase were prepared by adding the enzyme solution (in pH 7.0 25 mM phosphate buffer) to a solution of glutaraldehyde in the precipitant (in a 1:9, v/v ratio) under stirring at room temperature. After the crosslinking step, the mixture was centrifuged at 12,000 rpm for 10 min. The resulting CLEA was washed with phosphate buffer (pH 7.0 25 mM) and centrifuged at 12,000 rpm for 10 min. For esterification activity measurements the CLEA was washed with ethanol.

2.3. Hydrolytic activity assay of lipase

Hydrolytic activities of samples were measured under standard assay conditions (pH 7.0, 25 mM phosphate buffer including 2% tributryrin) by titration of the butyric acid that is released from substrate hydrolysis using an automatic titrimater (pH stat Metrohm). The reaction was carried out with continuous magnetic stirring at room temperature. One international unit of activity (IU) is defined as the amount of enzyme that catalyzes the release of 1 μmol butyric acid from tributyrin per minute under the standard assay conditions described above. The activity recovery in CLEAs is defined as: Total activity of the recovered CLEAs divided by the total activity of the free enzyme sample used.

Table 2
Effect of crosslinker concentration on activities of CLEAs.

Cross-linker (mM)	CLEA act (U)	(%) Activity recovery
5.0	137	6
12.5	153	6
25.0	533	22
40.0	496	21
50.0	390	16
65.0	229	9
80.0	222	9

Conditions: precipitant:enzyme soln. 9:1 (v/v), crosslinking time: 30 min, and [enzyme]: 50 mg/ml.

2.4. Synthetic activity assay of lipase

Synthetic activities were determined by incubating 5 ml of a 0.5 M solution of the alcohol and the acid in cyclohexane at 40 °C with mechanical stirring at 300 rpm. The amounts with the same units of hydrolytic activity of free enzyme and CLEAs were used for esterification activity. 0.5 ml samples were withdrawn and mixed with 10 ml of ethanol/acetone (1:1, v/v). Ester conversions were determined by measuring the amount of remaining acid by titration with 0.05 N KOH in the presence of phenolphthalein.

2.5. Thermal stability test

Enzymes were incubated in 25 mM, pH 7.0 phosphate buffer at three different temperatures (40 °C, 50 °C, 60 °C) for different periods of time and the remaining hydrolytic activities of the enzymes were measured.

2.6. Reusability

The reusability of CLEAs was tested by repeated use of the immobilized enzyme in tributyrin hydrolysis. CLEAs were recovered from reaction media by filtration with Whatman paper (pore size 0.2 μm).

3. Results and discussion

3.1. Optimization of CLEA preparation conditions

Initially we studied the effect of various precipitants on the activity recovery in the precipitated aggregates. A nine fold volume of precipitants was added to the enzyme solution (in 25 mM pH 7.0 phosphate buffer) and hydrolytic activities were determined for the redissolved enzyme aggregates. Saturated ammonium sulfate solution, ethanol, tert-butanol, acetone and iso-propanol were used as precipitants. Activity recoveries were calculated after precipitation and after subsequent crosslinking with glutaraldehyde. The results are shown in Table 1. The maximum activity recovery in the precipitation step was observed with iso-propanol but after crosslinking with glutaraldehyde the maximum activity recovery was obtained with the CrL lipase CLEAs prepared using ethanol as

Table 3
Effect of crosslinking time on activities of resulted CLEAs.

Crosslinking time (min)	CLEA activity (U)	(%) Activity recovery
15	518	24
30	513	24
45	584	27
60	473	22
75	427	20
90	333	15
120	330	15
150	293	14
180	210	10

Conditions: precipitant:enzyme soln. (v/v) 9:1, [glutaraldehyde]: 25 mM, and [enzyme]: 50 mg/ml.

Table 4
Effect of enzyme concentration on resulted CLEAs.

[Enzyme] (mg/ml)	Free enzyme activity (TU)	CLEA activity (TU)	(%) Activity recoveries
12.5	536	37	7
25	1072	198	18
50	2143	564	26
75	3216	530	16
100	4286	518	12

Conditions: precipitant:enzyme soln. (v/v) 9:1, crosslinking time: 45 min, and [glutaraldehyde]: 25 mM.

the precipitant. The observation that the optimum precipitant in the precipitation step is not necessarily the best precipitant for the overall CLEA formation can be explained as follows. The enzyme aggregates formed in the precipitation step may be in an induced conformation that is unfavorable for reaction but when they are redissolved in buffer they will exhibit their normal activity. On the other hand, when they are cross-linked they will remain in the unfavorable conformation and display a lower activity. In this case the activity recoveries of the redissolved enzyme aggregates and the CLEAs will be very different as can be seen in the examples shown in Table 1. Since ethanol gave the best overall results it was used as the precipitant in further experiments on CLEA preparation.

During the precipitation step enzyme aggregation is caused by decreasing enzyme solubility in the surrounding medium. The rate of aggregation can be important in the context of enzyme denaturation. During this step the enzyme structure is exposed to stress and aggregation with other enzyme molecules can have a stabilizing effect. The time interval between exposure of the enzyme molecule to the precipitant and stabilization of the structure by interaction and crosslinking with other protein molecules is likely to have a significant effect on the activity recovery. Therefore, we investigated the procedure for addition of the precipitant and cross-linker in more detail. CLEAs were prepared by adding precipitant and crosslinker consecutively or simultaneously. In the case of consecutive addition the enzyme solution (in buffer) was slowly added to the precipitant and allowed to aggregate for 30 min before adding the crosslinker. In the case of simultaneous addition, the enzyme solution was quickly added to the precipitant which already contained the crosslinker. However, we observed no significant difference between activity recoveries for these two methods, presumably because the precipitation step is instantaneous and much faster than the cross-linking step. Consequently, we chose the simultaneous preparation method for further studies because of its simplicity.

For the optimization of the crosslinking step, the effect of crosslinker amount and crosslinking time on activity recoveries of the resulting CLEAs was studied. The results are shown in Tables 2 and 3. A glutaraldehyde concentration of 25 mM and 45 min crosslinking time gave the highest activity recovery. As is evident from Table 2 the ratio of crosslinker to enzyme goes through an optimum. At lower ratios activity recoveries are low probably owing to insufficient crosslinking, affording a CLEA that is not stable towards leaching of free enzyme in water. If the ratio is too high too much crosslinking takes place resulting in a loss of the enzyme's flexibility that is necessary for its activity. Similarly, the activity recovery also decreases at long crosslinking times, presumably for the same reason.

As shown in Table 4, similar results were obtained, presumably for the same reasons, by varying the concentration of enzyme (12.5–100 mg/ml of crude enzyme preparation) at a constant amount of crosslinker and precipitant. The best result was obtained with 50 mg/ml enzyme concentration, 25 mM glutaraldehyde and 9 fold of precipitant.

Addition of bovine serum albumin (BSA) as a proteic feeder is known to facilitate CLEA preparation in cases where the protein concentration in the enzyme preparation is low and/or the enzyme activity is vulnerable to high concentrations of glutaraldehyde required to obtain aggregates [23,24]. We investigated the use of BSA and soybean protein in the preparation of the CrL CLEAs but observed no positive effect on activity recovery (data not shown).

The activation of lipases by additives such as surfactants, crown ethers or amines is well documented [25]. Surfactants preferentially interact with some of the binding sites of protein (enzyme) or form more powerful hydrophobic bonds than existing ones and thus change protein structure, thereby affecting its activity [26]. Lopez Serrano et al. observed the formation of hyperactive lipase CLEAs by pretreatment with surfactants. For example the CLEAs of the lipases from *Thermomyces lanuginosus* and *Rhizomucor miehei*

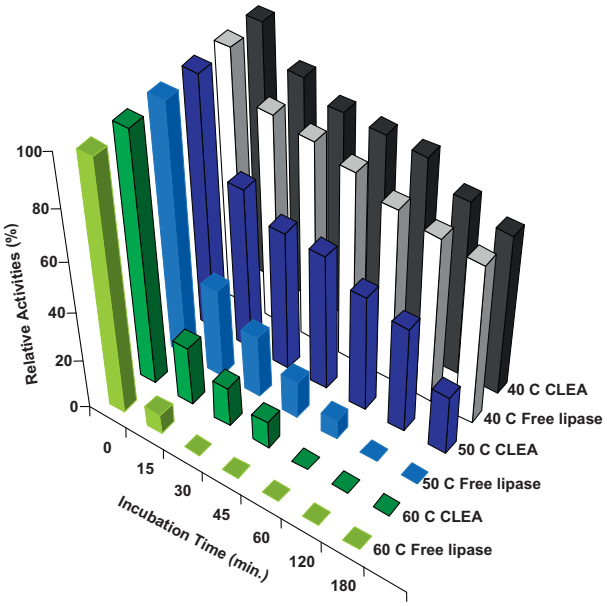

Fig. 1. Thermal stabilities of CrL and CrL-CLEA.

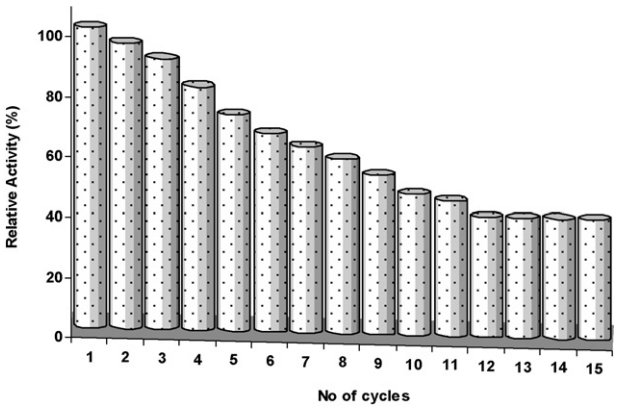

Fig. 2. Reusability of CLEAs.

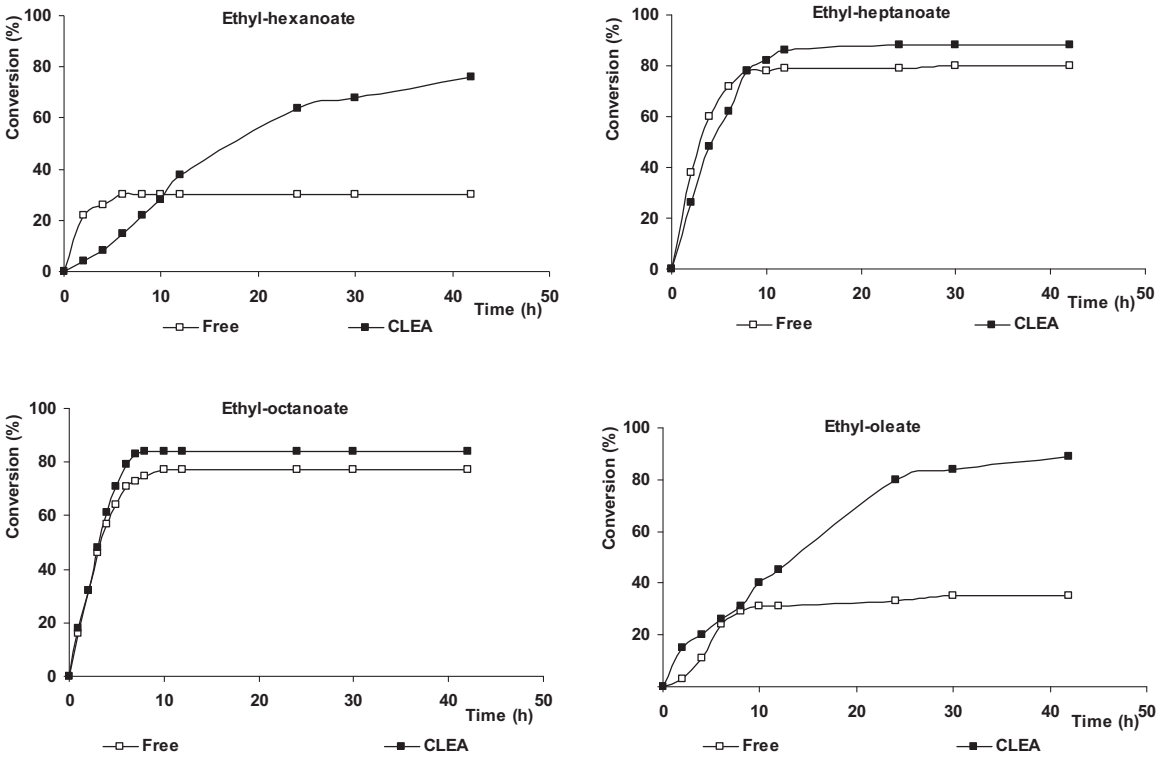

Fig. 3. Synthesis of ethyl esters of fatty acids.

made in the presence of SDS were 2–3 times more active, respectively, than the native enzymes. In contrast, the beneficial effects with lipases from *Pseudomonas alcaligenes*, *Aspergillus niger* and *C. rugosa* were slight [4]. We investigated the effect of pretreatment with the anionic surfactant, SDS, and the nonionic surfactants, Tween 80, Triton X-100 and Span 83, in the presence or absence of fatty acids, but we observed no increase in activity recoveries in the resulting CrL-CLEAs (data not shown).

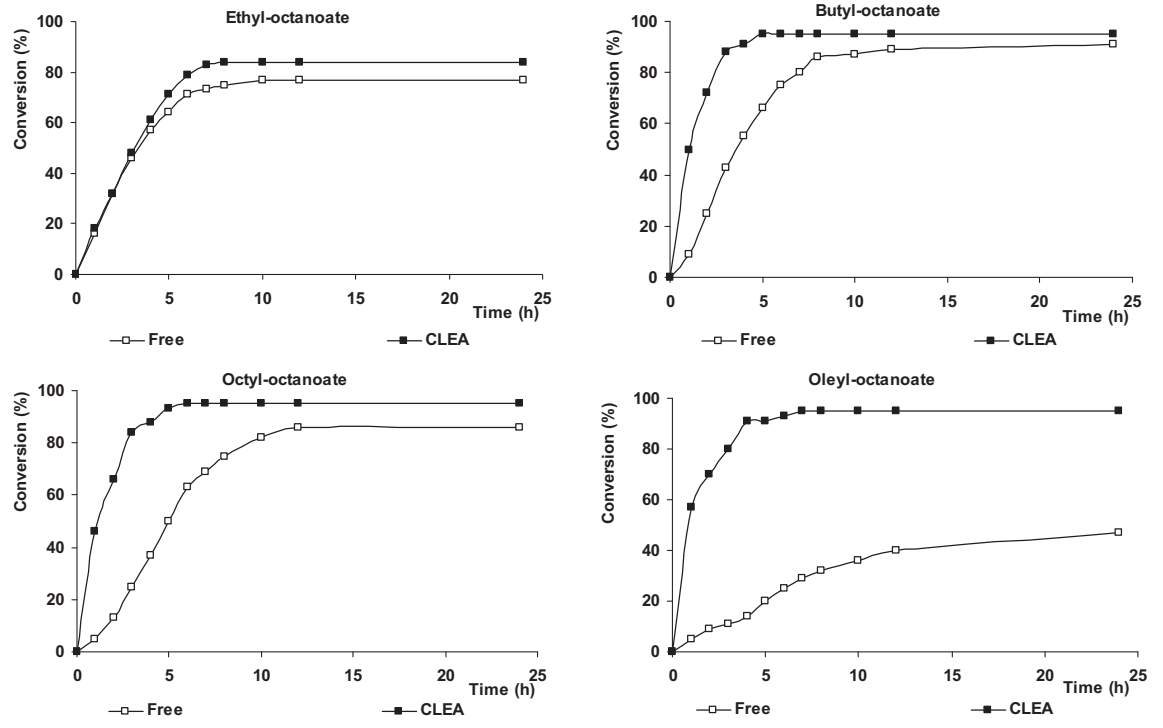

Fig. 4. Synthesis of different esters of octanoic acid.

3.2. Thermal stabilities

The thermostability of CLEAs was compared with that of the free enzyme at three different temperatures (40 °C, 50 °C, 60 °C). As shown in Fig. 1, the stability of the CLEA at 40 °C, was only slightly higher than that of the free enzyme but the difference was more pronounced at 50 °C and 60 °C.

3.3. Reusability

Results of recycle experiments are shown in Fig. 2. The activity decreased steadily up to 11 recycles but then it remained constant. For activity measurements the filter paper placed in new reaction medium to get all the enzyme from filter surface before reaction has started but accumulation of enzyme in the filter was a possibility. In a separate experiment the CLEAs were suspended in buffer (pH 7.0 25 mM phosphate) for 3 h (approximately equal to the time for 15 hydrolysis reactions) and after subsequent centrifugation activity measured at upper phase and the same activity loss was obtained with 15 cycle usage. The results suggest that the observed activity loss in the recycle experiments results from leaching of enzyme into the aqueous buffer and enzyme accumulation in the filter paper was not appreciable. After 11 recycles no more enzyme is leached and the CLEAs are stable. We note, however, that enzyme leakage is not necessarily an issue when using CLEAs in non-aqueous media.

3.4. Ester synthesis

We compared the free enzyme and CLEAs in the synthesis of esters from different fatty acids and alcohols in cyclohexane. The results of syntheses of ethyl esters of fatty acids of different chain length are shown in Fig. 3. The best conversions were obtained with heptanoic (C_7) and octanoic acids (C_8) maximum conversion being observed after approximately 10 h. In the case of shorter (hexanoic acid C_6) and longer (oleic acid C_{18}) fatty acids maximum conversions were much lower. For CLEAs maximum conversions were similar for all fatty acids: 76%, 88%, 84%, 89%, respectively, for hexanoic, heptanoic, octanoic and oleic acids but reaching maximum conversion with hexanoic and oleic acids took much longer. The higher conversions with heptanoic and octanoic acids can be the result of fast reaction of enzymes with these fatty acids. The explanation of higher conversions with CLEAs compared to free enzyme can be the result of enhanced stability of enzyme in organic solvents and better dispersibility of CLEAs while the free enzyme aggregated in time in organic solvent. Aggregation of the free enzyme can cause diffusion problems with the substrate and product, which can shift the equilibrium in favour of hydrolysis, rather than synthesis, because the former involves the relatively small nucleophile, water. Results of esterification reactions with octanoic acid and alcohols of different chain length are shown in Fig. 4. In all cases conversions were higher with the CLEA but the difference was more prominent in the case of oleyl alcohol where maximum conversions were 47% and 95%, respectively, for the free enzyme and the CLEA. Conformational changes of the enzyme structure after immobilization, making it more accessible for longer chain alcohols, could possibly explain the higher conversion of CLEAs with oleyl alcohol.

4. Conclusions

We have prepared cross-linked enzyme aggregates (CLEAs) of *C. rugosa* lipase (CrL) and showed that CrL CLEA has some advantages, such as thermal stability and good reusability. They also exhibited improved operational performance compared to the free enzyme in the synthesis of esters from a variety of alcohols and fatty acids possibly because of their enhanced stability and better dispersibility in organic solvents.

Acknowledgements

Authors gratefully acknowledge the support for this research provided by TUBİTAK (The Scientific and Technological Research Council of Turkey) and The Research Foundation of Ege University (BAP-Grant Number Sci 2007-011), and also thank Delft Technical University, Biocatalysis and Organic Chemistry group for hosting F. Kartal.

References

[1] L. Cao, L. van Langen, R.A. Sheldon, Curr. Opin. Biotechnol. 14 (2003) 387–394.
[2] L. Cao, F. van Rantwijk, R.A. Sheldon, Org. Lett. 2 (2000) 1361–1364.
[3] R.A. Sheldon, Biochem. Soc. Trans. 35 (2007) 1583–1587.
[4] P. Lopez-Serrano, C. Linqiu, F. van Rantwijk, R.A. Sheldon, Biotechnol. Lett. 24 (2002) 1379–1383.
[5] H.W. Yu, H. Chen, X. Wang, Y.Y. Yang, C.B. Ching, J. Mol. Catal. B: Enzym. 43 (2006) 124–127.
[6] H.R. Hobbs, B. Kondor, P. Stephenson, R.A. Sheldon, N.R. Thomas, M. Poliakoff, Green Chem. 8 (2006) 816–821.
[7] F. van Rantwijk, F. Secundo, R.A. Sheldon, Green Chem. 8 (2006) 282–286.
[8] V.S. Kumari, M.N. Gupta, Energy Fuels 21 (2007) 368–372.
[9] T.A. Ruiz, A.P. de los Ríos, F.J. Hernández, M.H.A. Janssen, R. Schoevaart, F. van Rantwijk, R.A. Sheldon, Enzyme Microb. Technol. 40 (2007) 1095–1099.
[10] P. Hara, U. Hanefeld, L.T. Kanerva, J. Mol. Catal. B: Enzym. 50 (2008) 80–86.
[11] L. Zhao, L. Zheng, G. Gao, F. Jia, S. Cao, J. Mol. Catal. B: Enzym. 54 (2008) 7–12.
[12] D. Ozdemirhan, S. Sezer, Y. Sönmez, Tetrahedron Asymmetry 19 (2008) 2717–2720.
[13] P. Gupta, K. Dutt, S. Misra, S. Raghuwanshi, R.K. Saxena, Bioresour. Technol. 100 (2009) 4074–4076.
[14] L. Wilson, G. Fernandez-Lorente, R. Fernandez-Lafuente, A. Illanes, J.M. Guisan, J.M. Palomo, Enzyme Microb. Technol. 39 (2006) 750–755.
[15] S.H. Krishna, N.G. Karanth, Catal. Rev. 44 (2002) 499–591.
[16] G. Langrand, N. Rondot, C. Triantaphylides, J.J. Baratti, Biotechnol. Lett. 12 (1990) 581–586.
[17] M. Mittelbach, J. Am. Oil Chem. Soc. 67 (1990) 168–170.
[18] F. Ergan, M. Trani, G. Andre, Biotechnol. Bioeng. 35 (1990) 195–200.
[19] P. Dominguez de Maria, J.M. Sanchez-Montero, J.V. Sinisterra, A.R. Alcantara, Biotechnol. Adv. 24 (2006) 180–196.
[20] M.H. Guncheva, D. Zhiryakova, Biotechnol. Lett. 30 (2008) 509–512.
[21] M. Kim, J.M. Park, H.J. Um, D.H. Lee, K.H. Lee, F. Kobayashi, Y. Iwasaka, C.S. Hong, J. Min, Y.H. Kim, J. Basic Microbiol. 50 (2010) 218–226.
[22] S. Shah, M.N. Gupta, Bioorg. Med. Chem. Lett. 17 (2007) 921–924.
[23] S. Shah, A. Sharma, M.N. Gupta, Anal. Biochem. 351 (2006) 207–213.
[24] B.S. Aytar, U. Bakir, Process Biochem. 43 (2008) 125–131.
[25] F. Theil, Tetrahedron 56 (2000) 2905–2919.
[26] J.H. Fendler, E.J. Fendler, Catalysis in Micellar and Macromolecular Systems, Academic Press, New York, 1975.