Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 29 (2004) 163–171
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Stabilization of an intracellular *Mucor circinelloides* lipase for application in non-aqueous media

Miroslawa Szczesna-Antczak [a,*], Tadeusz Antczak [a], Malgorzata Rzyska [a], Zofia Modrzejewska [b], Justyna Patura [a], Halina Kalinowska [a], Stanislaw Bielecki [a]

[a] *Faculty of Biotechnology and Food Science, Institute of Technical Biochemistry, Technical University of Lodz, 4/10 Stefanowskiego Str., 90-924 Lodz, Poland*
[b] *Department of Environmental Engineering Systems, Faculty of Process and Environmental Engineering, Technical University of Lodz, 213 Wolczanska Str., 90-924 Lodz, Poland*

Received 3 July 2003; received in revised form 28 January 2004; accepted 2 February 2004

Available online 9 April 2004

Abstract

Different methods for stabilization of *Mucor circinelloides* lipase, facilitating its application in organic solvents were tested. Lipase was either isolated from the mycelium and immobilized on solid carriers (derivatives of cellulose, diatomaceous earth, modified porous glass) or immobilized in situ in the mycelium pellets and stabilized. The immobilized enzyme preparations were used for synthesis of sucrose, glucose, butyl and propyl oleates and caprylates, carried out in petroleum and di-*n*-pentyl ethers. Immobilized preparations of either crude or purified lipase isolated from the mycelium were at least 4–6 times less effective in sucrose esters synthesis than mycelium-bound lipase preparations. Lipase preparation with the highest synthetic activity was obtained by cross-linking of *M. circinelloides* mycelium pellets with glutardialdehyde (operational stability in sucrose caprylate synthesis was 94% after 4 runs (24 h each), and caprylic acid conversion was 91–85%). The best method for production of mechanically durable biocatalyst, which efficiently catalyzed sucrose esters synthesis, was found to be entrapment of the mycelium-bound lipase in polyvinyl pyrrolidone-containing chitosan beads solidified with hexametapolyphosphate.

Keywords: Immobilization; Membrane-bound lipase; *Mucor circinelloides*

1. Introduction

Lipases are frequently applied in organic solvents and therefore various methods of their stabilization, including immobilization have been established [1–8]. Also genetic modifications of bacteria, leading to production of cell wall-anchored lipases and giving novel-biocatalysts have been described [9,10].

One of effective producers of intracellular cell membrane-bound lipase is *Mucor circinelloides* strain, which grows well in triacylglycerol-containing culture media [11–14]. Several other intracellular lipases, derived from *Mucor javanicus* [15], *Rhizopus arrhizus* [15–19], *R. oryzae* [20], *Pseudomonas fragi* [21], *Rhizopus chinesis* [22], *Penicillium cyclopium* [19], *Aspergillus niger* [23], and *A. flavus* Link [24,25] have been reported to date. Properties of the *M. circinelloides* lipase have been described elsewhere [11–13,26–31]. Due to high activity (both in triacylglycerols hydrolysis and synthesis of esters of fatty acids and aliphatic alcohols, glycerol and saccharides) and high stability in apolar organic solvents (it remains active at 100 °C in a hydrophobic solvent [12]), it can be applied in many processes, particularly for synthesis of various esters. Its immobilization, enabling repeated usage, would reduce the costs of its application.

Abbreviations: GA, glutardialdehyde; HMPP, sodium hexametapolyphosphate; MBL, mycelium-bound lipase preparation; *p*-NPA, *p*-nitrophenyl acetate; *p*-NPP, *p*-nitrophenyl palmitate; PVA, poly(vinyl alcohol); PVP, polyvinylpyrrolidone; TPP, tripolyphosphate; A_H, hydrolytic activity, in olive oil hydrolysis; A_S, synthetic activity, in ester synthesis; A_E, esterolytic activity towards *p*-NPA

* Corresponding author. Tel.: +48-42-631-3434; fax: +48-42-631-3402.
E-mail address: mirszcz@mail.p.lodz.pl (M. Szczesna-Antczak).

1381-1177/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2004.02.010

2. Materials and methods

2.1. Chemicals

Chitosan from crab shells (M_w of 458 000, DH of 77.6, moisture of 12.8%) was from Fluka Co.; octanoyl-, octadecanoyl-, and unmodified porous glass beads from J.T. Baker; PVP ($M_w \sim 25\,000$); diatomaceous earth (Hyflo Super Cell) from Serva; micro-granulated cellulose from Whatman Ltd.; γ-amino-propyl-silane, PVA (M_w of 72 000; DP of 1600; degree of hydrolysis of 97.5–99.5), Triton X-100, hexane, HMPP, TPP, di-*n*-pentyl and petroleum ethers from Fluka Co.; oleic and caprylic acids from Merck; butanol, and propanol from BDH; molecular sieve (4 Å) from Supelco; *p*-NPA, sodium alginate, and GA from Sigma Co. All other reagents were analytical grade.

2.2. Microorganism

M. circinelloides strain from the Institute of Technical Biochemistry (ITB) of the Technical University of Lodz microbial culture collection, was cultured for 72 h at 30 °C in a medium containing 5.9% (v/v) corn steep liquor and 2.7% (v/v) olive oil. The mycelium (suspension or pellets) was harvested by filtration, washed with water, and used for production of immobilized lipase preparations (see Sections 2.4–2.6).

2.3. Crude preparations of membrane-bound lipase

M. circinelloides mycelium (Section 2.2) was de-fatted with acetone (washed three times with 100 ml of acetone per 100 g of wet mycelium), dried at room temperature, and ground in a mortar, thus yielding the crude powdered MBL preparation (no. 1), particles of about 3–10 μm in diameter. This material was used for extraction of lipase (Section 2.4), and for the entrapment of mycelium-bound lipase in chitosan (Section 2.5).

2.4. Immobilization of soluble lipase preparations

The lipase was extracted from the crude preparation no. 1 (Section 2.3) with sodium cholate [12], dialyzed and lyophilized to obtain crude preparation of soluble lipase (preparation no. 2) which was purified [12] and lyophilized (preparation no. 3). Both preparations of soluble lipase (nos. 2 and 3) were immobilized on various carriers by using the methods described in Sections 2.4.1–2.4.4.

2.4.1. Immobilization of soluble lipase on cellulose derivatives

Cellulose palmitate and octate beads (10 g) [32], were washed with acetone, and suspended in Tris–HCl buffer (50 ml), pH 7.2, and lipase solution (10 ml of 1 and 0.1% (w/v) of the soluble lipase preparations 2 and 3, respectively) in the same buffer was added. This mixture was incubated for 1 h at 25 °C, with stirring. The immobilized preparations were harvested by filtration and dried at room temperature.

2.4.2. Immobilization of soluble lipase on diatomaceous earth

Diatomaceous earth (10 g) was washed with water and suspended in 50 ml of Tris–HCl buffer (pH 5.1), and lipase preparation no. 2 (0.1 g) or no. 3 (0.01 g) and acetone (60 ml) were added (at 4 °C). Further steps were as in Section 2.4.1.

2.4.3. Immobilization of soluble lipase on octanoyl-, octadecanoyl- and silanized glass beads

Immobilization was carried out under the same conditions as in Section 2.4.1. Glass beads (porosity of 2000 Å) were treated with γ-amino-propyl-silane and GA [33]. The immobilization of lipase on octadecanoyl- and octanoyl-glass was carried out at 4 °C for 4 h.

2.5. Entrapment of crude preparations of membrane-bound lipase in chitosan

Encapsulation of the mycelium-bound lipase in chitosan beads was achieved by phase inversion. Polymer solution (2−4.5% (w/w) in 1−1.8% acetic acid) contained lipase (preparation no. 1) in the weight ratio of 0.5:1 and 1:1, with respect to chitosan. PVP was added to the mixture at a chitosan:PVP ratio of 40:60 to enhance its hydrophilic character. The beads were formed by coagulation in 5% TPP dissolved in Tris–HCl buffer (pH 7.0) for 75 min, and in 3.5 and 7% HMPP aqueous solutions, for 24 h and 15 min, respectively. Some batches of beads were additionally lyophilized.

2.6. Preparations of stable M. circinelloides mycelium pellets

The pellets (1–2 mm in diameter) of *M. circinelloides* mycelium (from 72 h culture in optimal medium, inoculated with sporangiospores suspended in 0.1% Triton X-100), were washed with water and stabilized by using the following methods: washing three times with acetone (Sample 1), treatment with 1% alginate, 1% $CaCl_2$ (24 h) and acetone (Sample 2), treatment with 1% GA (pH 3, 4 °C, 1 h), 1% glycine (24 h), and finally with acetone (Sample 3), lyophilization (Sample 4) and treatment with 4% PVA (24 h), 1% GA (pH 3, 4 °C, 1 h), 1% glycine (24 h) and acetone (Sample 5). All these preparations (stabilized pellets of mycelium, approximately 0.5 mm in diameter) were applied for reactions of sucrose ester synthesis.

2.7. Determination of the activities of lipase preparations

The activities of lipase preparations towards *p*-NPA (esterolytic, A_E), and olive oil (hydrolytic, A_H) were determined by standard methods. Details are presented in Section 3. The concentration of *p*-NP released by enzyme was measured at $\lambda = 399$ nm (molar extinction coefficient for *p*-NP was

$1.32 \times 10^4\ M^{-1}\ cm^{-1}$). The amount of acid liberated during olive oil hydrolysis was determined by titration up to pH 10. The specific activity of lipase was expressed in micromoles of *p*-NP (or micromoles of fatty acids) released by 1 g of lipase preparation per 1 min (or $\mu kat\ g^{-1}$).

The ester synthesis was performed in di-*n*-pentyl and petroleum ether in tightly closed Pyrex vials (25 ml) at 30 or 50 °C on a reciprocal shaker (180–220 rpm). Details are presented in the figures and tables. The degree of ester formation was determined by acid titration with 0.05 M NaOH in 96% ethanol. One unit of specific synthetic activity (A_S) was defined as an amount of enzyme preparation (lipase-containing dried mycelium, preparation no. 1; crude preparation of soluble lipase, preparation no. 2; or purified lipase, preparation no. 3), which catalyzed the synthesis of 1 μmol of ester per 1 min (or as μkat per 1 g).

3. Results and discussion

Different methods for immobilization of *M. circinelloides* intracellular lipase in order to enhance its stability in organic solvents were tested. Fourteen to 20 grams of de-fatted and dried mycelium (preparation no. 1; Section 2.3) bearing the mycelium-bound enzyme was obtained from 1 l of the culture broth. Lipase in a soluble form was extracted from mycelium with sodium cholate and lyophilized (preparation no. 2). In the last step the enzyme was purified 33-fold by affinity chromatography (Section 2.4). It was estimated that the homogeneous lipase (preparation no. 3) (molecular mass of 42 kDa [12]) constituted approximately 0.57% (w/w) of de-fatted and dehydrated mycelium.

3.1. Activity of soluble lipase immobilized on solid carriers

The lyophilized, crude and purified preparations (no. 2 and no. 3, respectively) of soluble lipase were immobilized on mechanically durable carriers (see Sections 2.4.1–2.4.6). Hydrophobic supports (except the diatomaceous earth) that were stable in organic solvents were chosen. The immobilization of crude (Table 1, Exp. 1) and purified (Table 1, Exp. 2) lipase gave biocatalysts with synthetic activity 6–50, and 4–260 times lower, respectively, as compared to the MBL preparation (no. 1). The lipase adsorbed on diatomaceous earth was the most efficient biocatalyst, and the application of the lipase covalently linked to porous glass, which was the most mechanically stable carrier, gave the lowest yield. The value of optimum log *P* of reaction mixture depended on ester and lipase preparation, e.g., petroleum ether (log *P* 3.2), and benzene (log *P* 2.0) were the most suitable solvents for propyl oleate synthesis catalyzed by the MBL and purified lipase immobilized on diatomaceous earth, respectively (Fig. 1). Probably, due to the unnatural and more hydrophobic microenvironment of enzyme, the immobilized preparation required less hydrophobic organic solvents for efficient ester synthesis as compared to the mycelium-bound lipase. The substrate specificity of the lipase immobilized on diatomaceous earth was markedly different from that of the membrane-bound enzyme, which preferably synthesized oleic acid esters. The first enzyme displayed the highest activity in synthesis of sucrose caprylate (Table 2). Noteworthy, the *M. circinelloides* MBL preparation displayed the highest (off all the examined preparations) specific synthetic activity (A_S). It was approximately 4 and 10 times larger than the activity of homogeneous lipase immobilized on

Table 1
Activity of crude and purified lipase preparations immobilized on various solid carriers

Exp. no.	Lipase	Carrier	A_S ($\mu kat\ g^{-1}$)	A_H ($\mu kat\ g^{-1}$)
1[a]	Soluble crude preparation (no. 2)	Cellulose octate	0.95	0.54
		Cellulose palmitate	0.84	0.24
		Diatomaceous earth	0.73	0.82
		Octanoyl-modified glass	0.82	0.68
		Octadecanoyl-modified glass	0.67	0.50
		Silanized glass beads	0.11	0
	Membrane-bound lipase (no. 1)		6.12	7.78
2[b]	Purified lipase (no. 3)	Soluble in water	4.22	3380.2
		Soluble in benzene	69.90	534.6
		Cellulose octate	96.60	0.13
		Cellulose palmitate	2.67	0.06
		Diatomaceous earth	176.10	0.03
		Glass (all preparations)	0.05	0
	Membrane-bound lipase (no. 1)		706.8	1353
			4.06*	7.78*

[a] A_S: synthetic activity per 1 g of the biocatalyst determined in sucrose caprylate synthesis. Reaction conditions: caprylic acid (1 mmol), and sucrose (1 mmol) in di-*n*-pentyl ether saturated with water (5 ml), biocatalyst (50 mg), 50 °C; 210 rpm, 10 min; A_H: hydrolytic activity, determined towards olive oil emulsion (Section 2.7).

[b] A_S: specific synthetic activity per 1 g protein (or in asterisk: per 1 g of mycelium), determined in propyl oleate synthesis. Reaction conditions: oleic acid (1 mmol), and 1-propanol (1 mmol) in petroleum ether (5 ml), immobilized (50 mg) or purified (0.1 mg) lipase preparations, 30 °C; 180 rpm, 20 min; A_H: hydrolytic activity of lipase towards glycerol trioleate.

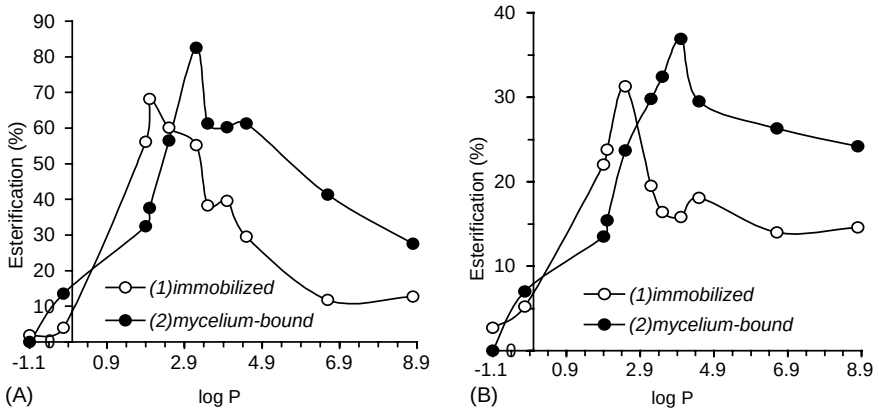

Fig. 1. The effect of polarity of organic solvent on the efficiency of propyl (A) and glycerol (B) oleate synthesis, catalyzed by two lipase preparations: the mycelium-bound lipase (2) and purified lipase immobilized on diatomaceous earth (1). Reaction conditions: oleic acid (1 mmol) and alcohol (1 mmol) in petroleum ether (5 ml), lipase preparations (50 mg), 30 °C; 180 rpm, 18 h.

diatomaceous earth and that of the lipase dissolved in toluene, respectively (Table 1).

The enzyme immobilized on hydrophobic supports displayed a surprisingly low activity. Guisán et al. [34] observed that the immobilization of some lipases, such as the extracellular *M. javanicus* lipase, on hydrophobic carriers with a large specific area resulted in their *hyper-activation*. It agreed with the hypothesis that the lipases recognize a hydrophobic surface as a stable interface, compatible to their hydrophobic region around the catalytic site in its open, active state. Adsorption of the lipase on the support protects the stable and highly active structure of the catalytic site [34–36]. The *Mucor* lipase was believed to possess the 3D structure, which provided the highest activity in the cell membrane-bound form. Its isolation and immobilization on the carriers (even hydrophobic) resulted in conformational changes, similar to that caused by dissolving in toluene [12], and disturbed an access of the substrate to the active site, which was closed by the lid.

3.2. Synthetic activity of stabilized pellets of Mucor mycelium

Molecules of the intracellular *Mucor* lipase remain anchored in the mycelium after extraction of acetone-soluble substances [13,26]. This in situ immobilization was

Table 2
Comparison of ester synthesis catalyzed by the purified *M. circinelloides* lipase immobilized on diatomaceous earth or by membrane-bound lipase

Alcohol/sugar	Lipase preparation (esterification, %)					
	Purified, immobilized on diatomaceous earth			Membrane-bound		
	Caprylic acid	Palmitic acid	Oleic acid	Caprylic acid	Palmitic acid	Oleic acid
Ethanol	22.2	33.8	26.5	90	92	94
1-Propanol[a]	66.4	83.5	55.1	80	83	96
	38.2	*43.2*	*45.3*			
1-Butanol[a]	80.6	80.1	57.1	96	97	99
	46.5	*46.0*	*47.4*			
2-Pentanol	11.7	4.0	10.0	18	25	32
2-Methyl-1-butanol	11.0	1.1	10.6	83	88	92
2-Methyl-2-butanol	6.4	0	5.7	6	9	8
1-Hexanol	60.4	57.9	39.8	80	85	98
Glycerol	41.2	23.7	19.6	30	23	49
	28.5	*16.1*	*15.9*			
Hexadecenol[a]	52.5	45.4	20.5	89	92	90
	34.3	*33.5*	*30.9*			
Benzyl	37.9	33.4	22.3	76	80	83
Sucrose[a]	*21.1*	*17.3*	*18.5*	78	76	79
Glucose	–	–	–	80	77	81

Reaction conditions: acid (1 mmol), and alcohol (1 mmol) in petroleum ether (5 ml), immobilized purified lipase or MBL (50 mg of each), 30 °C (only sugar esters were synthesized at 50 °C in di-*n*-pentyl ether saturated with water); 180 rpm, 18–20 h. Two grams of molecular sieve 4 Å were added to MBL-containing reaction mixtures.

[a] The results of ester synthesis catalyzed by the purified lipase (0.1 mg of pure protein dissolved in 5 ml of toluene) are in italics. The other reaction conditions as above.

exploited for production of preparations of *M. circinelloides* lipase that were very active and stable in organic solvents, as described in [11,12]. *M. circinelloides* mycelium particles displayed activities of 4–6 μkat g^{-1} in synthesis of sucrose caprylate and butyl oleate, carried out in di-*n*-pentyl and petroleum ether, respectively (Table 1), and efficiently catalyzed synthesis of other esters (Table 2). This MBL preparation was very stable in organic solvents. However, centrifuging at 10 000 × *g* was necessary to separate the powdered form of this preparation from reaction mixture. Neither it could be applied as a bed for column reactor. Therefore, the lipase preparations in the form of granulated mycelium were obtained. After being cultivated under conditions presented in Section 2.6, the *Mucor* pellets were harvested and stabilized in order to enhance their mechanical durability (Section 2.6). After washing with organic solvent (acetone, di-*n*-pentyl and petroleum ether mixture), these stabilized pellets of *Mucor* mycelium were used for sucrose ester synthesis (Fig. 2).

The most efficient method for stabilization of the pellets containing the mycelium-linked *M. circinelloides* lipase was their treatment with GA (Fig. 2, Sample 3). Cross-linking of the pellets with calcium alginate (Sample 2) and poly(vinyl alcohol)-acetals [37] (Sample 5) decreased the yield of ester synthesis, presumably because these hydrophilic polymers adsorbed more water. The influence of the hydrophilic character of PVA on the dynamics of ester synthesis by entrapped *M. circinelloides* lipase was discussed elsewhere [14]. In the presence of the molecular sieve (4 Å) in reaction mixture the *M. circinelloides* pellets cross-linked with PVA and GA (Sample 5) revealed both high synthetic activity (∼3.3 μkat g^{-1}) and operational stability ($\tau_{1/2}$ of 170 h) in the sucrose caprylate synthesis. The stabilized pellets of mycelium can be used as a biocatalyst in column and fluidized-bed reactors. However, the intensive agitation of the reaction mixture significantly reduced the pellet life. Therefore the method of an entrapment of the membrane-bound *M. circinelloides* lipase in a highly porous and mechanically durable carrier was also developed.

3.3. Lipase immobilization in chitosan–mycelium composites

In earlier experiments, the membrane-bound *M. circinelloides* lipase (powdered preparations, no. 1) was entrapped in carragenan, silica aerogel matrix [26,28], and in PVA-cryogels [14,38,39]. The lipase immobilized in these polymers exhibited good stability and activity in hydrolysis of triacylglycerols, *p*-NPA and *p*-NPP, and in synthesis of different aliphatic alcohols esters, but their efficiency in synthesis of saccharide esters was not satisfactory (the maximum yield of the reaction did not exceed 5%). Therefore chitosan was tested as a potential lipase carrier. Its gel was formed by several methods including the cross-linking and ionotropic gelation with GA, NaOH, TPP, and HMPP. The immobilized preparations were used for *p*-NPA hydrolysis (results not presented). The fastest method giving the most active beads was found to be the phase inversion, coupled with ionic chitosan gel forming with polyphosphate salts. The fast gel formation caused the least enzyme losses, usually observed in the first step of gelling, when the beads are not hard enough. Furthermore, in the presence of polyphosphates (TPP and HMPP), chitosan gel was formed at neutral pH, which protects *M. circinelloides* lipase. More than 10 different variants of immobilization conditions were tested. Various concentrations of chitosan, lipase (MBL, preparation no 1), PVP, TPP and HMPP were used. The enzyme loading in beads, and their operational stability in organic solvents were optimized (Table 3). It was found that the weight of wet beads derived from the same volume of mycelium suspension and other reagents depended on a gel-forming compound. The application of HMPP for chitosan gel forming (Samples V−VIII, Table 3), gave hard beads with a tight structure, more mechanically

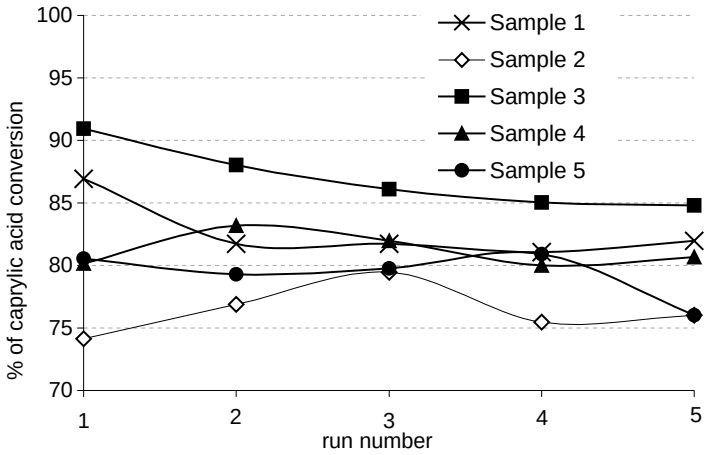

Fig. 2. Synthesis of sucrose ester using pellets of *M. circinelloides* mycelium stabilized by different methods. Reaction conditions: wet pellets (2 g), the weight before stabilization and dehydration, substrates (0.5 mmol of each), di-*n*-pentyl ether, molecular sieve 4 Å; 24 h, 50 °C; After each run, the pellets were washed with di-*n*-pentyl ether and used again. The beads, Samples 1–5, were prepared as described in Section 2.6.

Table 3
The effect of the method of production of *M. circinelloides* lipase immobilized in chitosan beads on lipase content and the activity in *p*-nitrophenyl acetate hydrolysis (A_E)

Sample	Gel-forming compound	Content (%)			A_E, *p*-NPA	Mass of beads produced from 100 ml of mixture (g)	MBL content in beads (%, w/w)
		CH	MBL	PVP			
I	5% TPP	2.0	1.0	–	0.110	Wet beads: 29.0	3.5
						Acetone-dehydrated: 18.2	~5.5
						Lyophilized: 2.8	~35.0
II	5% TPP	2.0	2.0	–	0.165	Wet beads: 29.5	6.8
						Acetone-dehydrated: 21.1	~9.5
						Lyophilized: 3.6	~57.0
III	5% TPP	2.5	2.5	–	0.098	Wet beads: 53.5	4.7
IV	5% TPP	2.5	2.5	1.5	0.120	Wet beads: 64.2	3.9
V	3.5% HMPP	2.5	2.5	–	0.169	Wet beads: 16.7	15.0
VI	3.5% HMPP	2.5	2.5	1.5	0.173	Wet beads: 31.3	8.0
VII	3.5% HMPP	4.5	4.5	2.7	0.179	Wet beads: ~54.0	7.6
						Acetone-dehydrated: 34.8	~11.7
						Lyophilized: 8.9	~45.8
VII	7% HMPP	4.5	4.5	2.7	0.185	Wet beads: ~56.0	8.0

CH: chitosan, A_E: expressed in (μmol *p*-NP min^{-1} g^{-1} beads); reaction conditions: beads (10–30 mg), *p*-NPA solution (1.61 ml) in 0.05 M Tris–HCl buffer (pH 7.5) containing 2% acetonitrile, 20 min, 25 °C.

durable than the beads cross-linked with TPP. Successful application of HMPP for chitosan gel forming was also reported by Angelova and Hunkeler [40]. PVP increased the hydrophilic character of the chitosan support (Table 3, Samples IV, VI−VIII) and therefore after dehydration of the beads their structure became more open, and the diffusion resistance was weaker (as proved by differences in esterolytic activities, A_E, of the biocatalysts presented in

Table 4
Production of sucrose esters by membrane-bound *M. circinelloides* lipase entrapped in chitosan beads

Sample of beads[a]	Lipase preparation (mg)	No. of run	Beads dehydrated (% of caprylic acid conversion)	
			With acetone	By lyophilization
I	34.4	1	32.0	40.7
		2	29.7	29.3
II	67.7	1	32.0	74.7
		2	35.6	62.7
III	100.0	1	25.5	–
IV	100.0	1	26.2	–
V	100.0	1	39.3	–
VI	100.0	1	37.4	–
VII (a)[b]	107.0	1	60.6	77.4
		2	59.5	68.6
		3	56.3	54.5
		4	46.4	46.1
VII (b)[c]	110.0	1	58.3	75.8
		2	56.3	84.8
		3	45.8	76.8
		4	33.9	64.3
VIII	20.0	1*	32.3	–
		2*	31.9	–
		3	38.1	–
	60.0	1*	75.3	–
		2*	74.4	–
		3	76.0	–

Reaction conditions: sucrose and caprylic acid (0.5 mmol of each), di-*n*-pentyl ether (1.5 ml), petroleum ether (1.0 ml) (or in no. of runs indicated by asterisk: di-*n*-pentyl ether, 2.5 ml), beads of the biocatalysts (dried with acetone or lyophilized), 24 h, 50 °C. After each cycle of the reaction the beads were washed with the same mixture of organic solvents, which was used as the reaction medium.

[a] Numbers of samples as under Table 3.

[b] The average bead diameter of 3–4 mm and weight of approximately 25 mg.

[c] The average bead diameter of 2 mm and weight of approximately 11 mg.

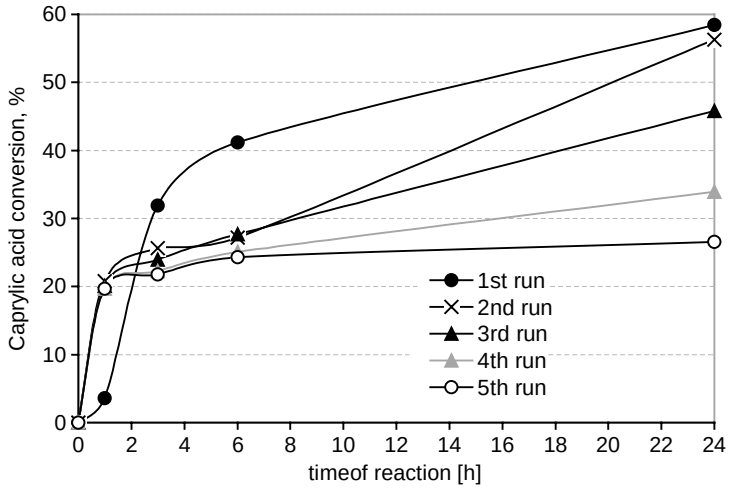

Fig. 3. The dynamics of sucrose ester synthesis in successive cycles of the process catalyzed by *M. circinelloides* lipase immobilized in chitosan. Reaction conditions: sucrose and caprylic acid (0.5 mmol of each) in di-*n*-pentyl ether (1.5 ml) and petroleum ether (1.0 ml), chitosan beads dried with acetone (Sample VII, Table 3) containing 33 mg of membrane-bound lipase, 24 h, 50 °C. After each cycle the beads were washed with the same solvent as applied for the reaction.

Table 3,). The maximal MBL loading in chitosan beads can exceed 45% (lyophilized beads, Sample VII, Table 3). Further increase in MBL content in the beads excluded their ionotropic gelling.

Water present in the beads should be removed prior to their application for synthetic reactions, particularly in the case of hydrophilic polymer matrices [41,42]. Chitosan gel can be dehydrated with acetone (dried by using the molecular sieve) or by lyophilization. These operations decreased the bead weight by 30–40 and 91–83%, respectively. The lyophilized beads had smaller size, whereas those dehydrated with acetone had unchanged dimensions. The beads dehydrated by using these two methods contained different amounts of water, and probably had different internal structure.

The samples of different MBL–chitosan beads were employed for sucrose caprylate synthesis (Table 4). High degree of caprylic acid conversion was achieved when beads were formed from the mixture containing 4.5% of MBL and 4.5% chitosan. The yield of sucrose caprylate synthesis was 78% of that of the reaction catalyzed by MBL preparation, but due to diffusion resistance, the reaction rate was lower and during the first 4–6 h only half of the maximum ester synthesis yield was achieved (Fig. 3). The rates of reaction catalyzed by the lipase stabilized in pellets of mycelium and by the MBL preparation were higher. In this latter case, as much as approximately 95% of the product were synthesized for 2–6 h (dependently on synthesis conditions [13]). The application of the lyophilized MBL–chitosan beads brought about the higher synthesis yields in the initial process runs, as compared to the acetone-dehydrated beads of this polymer. However, the activity of the former biocatalyst was significantly decreased after the first process cycle, whereas the latter one displayed the better operational stability. The profiles of caprylic acid depletion are shown in Fig. 3. Synthetic activity of the chitosan beads rose from approximately 0.2 in the first run to 0.9 μkat g^{-1} in the next runs.

M. circinelloides lipase displayed the highest activity in sucrose ester synthesis in a mixture of di-*n*-pentyl ether and petroleum ether (3:2). The latter compound was found to protect the enzyme activity at 100 °C [12]. The lipase entrapped in chitosan beads displayed higher effectiveness in sucrose caprylate synthesis in this mixture of ethers than in pure di-*n*-pentyl ether but it was less stable in this medium (Fig. 4). High productivity of this ester synthesis required sufficient amount of the MBL entrapped in chitosan beads (more than 100 mg in the reaction mixture) (Fig. 4, Table 4).

Because chitosan beads can be easily harvested from the reaction mixture and used many times, the overall yield of

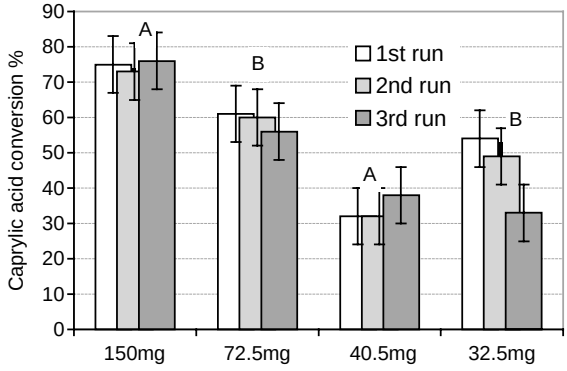

Fig. 4. The effect of the lipase content and reaction conditions on the yield of sucrose ester synthesis in the repeated-batch process, catalyzed by lipase immobilized in chitosan beads. Reaction conditions: sucrose and caprylic acid (0.5 mmol of each) in di-*n*-pentyl ether (2.5 ml) (A) or in di-*n*-pentyl ether (1.5 ml) and petroleum ether (1.0 ml) (B), the lipase-containing chitosan beads dried with acetone (Sample VII, Table 3), 24 h, 50 °C. After each cycle the beads were washed with the solvent applied as the reaction medium.

ester synthesis is increased and the costs of this biocatalyst application are reduced. Chitosan beads were chemically and mechanically durable and did not shrink in organic solvents, usually used for ester synthesis. Their destruction was not observed after several hundred hours of application at 50 °C and agitation (at 180–220 rpm). This high stability of the ionotropically cross-linked chitosan beads, containing approximately 50% powdered *Mucor* mycelium, suggests that their network was formed via the reaction of polyphosphate groups with amine groups of both chitosan [43], and proteins and polysaccharides of cell membranes, in which the *M. circinelloides* lipase was anchored. This tight association with matrix protects the lipase from leakage, observed in the case of immobilization of *Candida rugosa* lipase in chitosan gels cross-linked with TPP [44,45].

4. Conclusion

Isolation of the intracellular *M. circinelloides* lipase and immobilization on solid supports (cellulose palmitate and octate) gave preparations with markedly lower activity in synthesis of oleic and caprylic esters of propanol and sucrose, and weaker hydrolytic activity as compared to the MBL preparation (de-fatted and dehydrated mycelium). High activity of the latter preparation can be explained by the most favorable spatial arrangement of the lipase molecules, anchored in cell membranes that facilitates binding of substrates in the catalytic site of enzyme. None of the tested methods of the purified lipase immobilization gave rise to the similar arrangement, which seems to be optimal for catalysis. Physicochemical properties of carriers, which were different from the natural matrix, changed the enzyme's microenvironment, and had the negative impact on its catalytic activity.

The granulated preparation of *M. circinelloides* lipase derived by de-fatting, dehydration, and cross-linking with GA (sole or coupled with PVA) of mycelium pellets (derived from culture under optimized conditions) was found to be superior to other preparations of this enzyme due to higher synthetic and hydrolytic activity, and operational stability. Despite the relative mechanical durability this preparation was destroyed by vigorous agitation. Only the beads of *Mucor* lipase entrapped in ionotropically solidified chitosan (with HMPP) were stable under the latter conditions. Immobilization of membrane-bound lipase in chitosan solidified with HMPP gave mechanically durable biocatalyst beads, which were stable in organic solvents, and the enzyme did not leak into reaction mixtures. The best biocatalysts were formed from 4.5% chitosan solutions, containing approximately the same weight of membrane-bound lipase, and enriched with PVP which enhanced the hydrophilic character and porosity of the gel. Application of this biocatalyst for sucrose caprylate synthesis provided 70% conversion of the acid after more than 10 h of reaction. Due to the satisfactory enzyme-holding properties, and high stability in organic solvents, chitosan–HMPP gels seem to be better candidates for immobilization of lipases than chitosan–TPP and PVA-cryogels, and gels of other polysaccharides, such as alginate and carrageenan.

Acknowledgements

The authors gratefully acknowledge the financial support of the State Committee for Scientific Research (KBN) for this work under Grant PBZ KBN 021/PO6/99/22.

References

[1] R.J. Kazlauskas, U.T. Bornscheuer, in: D.R. Kelly (Ed.), Biotechnology, Biotransformation, vol. 8a, 2nd ed., Wiley/VCH, Weinheim, 1998, p. 37.
[2] K.E. Jaeger, M.T. Reetz, Trends Biotechnol. 16 (1998) 369.
[3] A. Schmid, J.S. Dordick, B. Hauer, A. Kiener, M. Wubboltz, B. Witholt, Nature 409 (2001) 258.
[4] B.G. Davis, V. Boyer, Nat. Prod. Rep. 18 (2001) 618.
[5] A.F. Hsu, K. Jones, T.A. Foglia, W.N. Marmer, Biotechnol. Appl. Biochem. 36 (2002) 181.
[6] M.W. Christensen, L. Andersen, T.L. Husum, O. Kirk, Eur. J. Lipid Sci. Technol. 105 (2003) 318.
[7] F.-Ch. Huang, Ch.-H. Ke, Ch.-Yu. Kao, W.-Ch. Lee, J. Appl. Polym. Sci. 80 (2001) 39.
[8] N.S. Dosanjh, J. Kaur, Biotechnol. Appl. Biochem. 36 (2002) 7.
[9] A. Tsuchiya, G. Kobayashi, H. Yamamoto, J. Sekiguchi, FEMS Microbiol. Lett. 176 (1999) 373.
[10] G. Kobayashi, J. Toida, T. Akamatsu, H. Yamamoto, T. Shida, J. Sekiguchi, J. Biosci. Bioeng. 90 (2000) 422.
[11] T. Antczak, D. Hiler, A. Krystynowicz, S. Bielecki, E. Galas, J. Mol. Catal. B: Enzymatic 11 (2001) 1043.
[12] T. Antczak, J. Graczyk, M. Szczesna-Antczak, S. Bielecki, J. Mol. Catal. B: Enzymatic 19–20 (2002) 287.
[13] T. Antczak, D. Hiler, A. Krystynowicz, M. Szczesna, S. Bielecki, E. Galas, Prog. Biotechnol. 17 (2000) 221.
[14] M. Szczesna-Antczak, T. Antczak, M. Rzyska, S. Bielecki, J. Mol. Catal. B: Enzymatic 19–20 (2002) 261.
[15] C.E.L. Shaw, J.A. Blain, J.D.E. Patterson, FEMS Microb. Lett. 3 (1979) 223.
[16] J.D.E. Patterson, J.A. Blain, C.E.L. Shaw, R. Todd, G. Bell, Biotechnol. Lett. 1 (1979) 211.
[17] T. Knox, K.R. Cliffe, Process. Biochem. 19 (1984) 188.
[18] C. Ganet, Ann. N.Y. Acad. Sci. 613 (1990) 600.
[19] V. Legier, L.C. Comeau, Appl. Microbiol. Biotechnol. 37 (1992) 732.
[20] M. Essamri, V. Deyris, L. Comeau, J. Biotechnol. 60 (1998) 97.
[21] C. Schuepp, S. Kermasha, M.C. Michalski, A. Morin, Process Biochem. 32 (1997) 225.
[22] T. Nakashima, H. Fukada, S. Kyotani, H.J. Morikawa, J. Ferment. Technol. 66 (1988) 71.
[23] K. Mahmoud, M. Tahoun, F. El-Kady, A.A. Wahba, Microbios 47 (1986) 45.
[24] K. Long, H.M. Ghazali, A. Ariff, Y.C. Man, Ch. Bucke, Biotechnol. Lett. 20 (1998) 369.
[25] K. Long, H.M. Ghazali, A. Ariff, Ch. Bucke, J. Am. Oil Chem. Soc. 74 (1997) 1121.
[26] A. Jarzêbski, A. Lachowski, J. Lorenc, J. Malinowski, T. Antczak, S. Bielecki, E. Galas, Patent RP 175 386 (1994).
[27] U. Antczak, J. Góra, T. Antczak, E. Galas, Enzyme Microb. Technol. 13 (1991) 589.

[28] T. Antczak, J. Morowiec-Bialon, S. Bielecki, A. Jarzebski, J. Malinowski, A. Lachowski, E. Galas, Biotechnol. Techn. 11 (1997) 9.
[29] E. Galas, T. Antczak, A. Krystynowicz, Patent RP 150 601 (1991).
[30] E. Galas, T. Antczak, U. Antczak, J. Góra, Patent RP 153 064 (1990).
[31] E. Galas, A. Krystynowicz, T. Antczak, Patent RP 162 079 (1993).
[32] Z.Ch. Dijorow, A.A. Diukczuwene, A.B. Paulukomis, Chim. Prir. Sojed. 5 (1978) 624.
[33] P.J. Robinson, P. Dunnill, M.D. Lilly, Biochim. Biophys. Acta 242 (1971) 659.
[34] J.M. Guisán, A. Bastida, P. Sabuquillo, P. Armisen, R. Fernández-Lafuente, J. Huguet, Proceedings of the International Meeting on Lipases and Lipids: Structure, Specificity and Applications in Biocatalysis, Como, Italy, 1997, p. 36.
[35] A. Bastida, P. Sabuquillo, P. Armisen, R. Fernández-Lafuente, J. Huguet, J.M. Guisán, Biotechnol. Bioeng. 58 (1998) 486.
[36] V.M. Balcão, F.X. Malcata, Biotechnol. Bioeng. 60 (1998) 114.
[37] A.M. Araujo, M.T. Noves, W.M. Azavedo, G.G. Oliveira, D.L. Ferreira, R.A.P. Figueiredo, L.B. Carvalho, Biotechnol. Techn. 11 (1997) 67.
[38] M. Szczesna-Antczak, T. Antczak, S. Bielecki, Proceedings of the Second International Conference on Protein Stabilisation, Lisbon, 9–12 April 2000, p. 99.
[39] M. Szczesna-Antczak, T. Antczak, M. Rzyska, S. Bielecki, Proceedings of the Fifth International Symposium on Biocatalysis and Biotransformation, BioTrans, Darmstadt, 2–7 September 2001, p. 207.
[40] N. Angelova, D. Hunkeler, J. Biomater. Sci. Polymer Edn. 12 (2001) 1207.
[41] M. Essamari, V. Deyris, L. Comeau, J. Biotechnol. 60 (1998) 97.
[42] C. Ganet, Ann. N.Y. Acad. Sci. 613 (1990) 600.
[43] Y. Xu, Y. Du, Int. J. Pharm. 250 (2003) 215.
[44] S.S. Betigeri, S.H. Neau, Biomaterials 23 (2002) 3627.
[45] I.A. Alsarra, S.S. Betigeri, H. Zhang, B.A. Evans, S.H. Neau, Biomaterials 23 (2002) 3637.