Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 22 (2003) 307–313
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Purification and properties of thermostable lipase from a thermophilic *Bacillus stearothermophilus* MC 7

M. Kambourova*, N. Kirilova, R. Mandeva, A. Derekova

Department of Extremophilic Bacteria, Institute of Microbiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

Abstract

Extracellular thermostable lipase produced by the thermophilic *Bacillus stearothermophilus* MC 7 was purified to 19.25-fold with 10.2% recovery. The molecular weight of the purified enzyme determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was shown to be 62 500 Da. The purified enzyme expressed maximum activity at 75–80 °C and its half life was 30 min at 70 °C. The K_m and V_{max} were calculated to be, respectively, 0.33 mM and 188 μM min^{-1} mg^{-1} with *p*-nitrophenyl palmitate (*p*NPP) as a substrate. Enzyme activity was inhibited by divalent ions of heavy metals, thiol and serine inhibitors, whereas calcium ion stimulated its activity. The most advantageous method for immobilization was found to be ionic binding to DEAE Cellulose. The enzyme was able to hydrolyze both soluble and insoluble emulsified substrates and was classified as a lipase, expressing some esterase activity as well.

Keywords: Thermophilic *Bacillus*; Thermostable lipase; Purification; Characterization; Immobilization

1. Introduction

Lipases (EC 3.1.1.3) are an important group of biotechnologically valuable enzymes as they are able to catalyze reactions at the oil–water interface. Their importance and application in stereospecific synthesis has increased in recent years. The industrial demand for lipases well active at high temperature continues to stimulate the search for microorganisms producers of thermostable enzymes. The advantages of running bioprocesses at elevated temperatures, favorable changes in most physical properties of fats in increased temperature, and increased stability of thermostable lipases to organic solvents determine the increased interest to new thermostable enzymes for new applications. Thermostable enzymes are usually derived from thermophilic strains, which may be expected to produce intrinsically more heat-stable enzymes than their mesophilic counterparts. A few numbers of thermophilic lipase-producing bacteria have been described in the last decade [1–8]. As reported in our previous papers [9,10] *Bacillus* sp. MC 7 originally isolated from Bulgarian hot spring was between first reported thermophilic producers.

The aim of the present investigation was to purify and characterize the thermostable lipase produced by that thermophilic strain, additionally classified by 16S rDNA as *Bacillus stearothermophilus*.

2. Experimental

2.1. Production of lipase

B. stearothermophilus MC 7 was cultivated in a jar fermentor (New Brunswick Co., Edison, NY) with

* Corresponding author. Tel.: +359-2-9793183; fax: +359-2-700109.
E-mail address: margikam@bas.bg (M. Kambourova).

1381-1177/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S1381-1177(03)00045-6

working volume 1.2 l for 18 h. The medium was composed of 0.5% soybean flour and 0.1% olive oil. The fermentation conditions were as follows: temperature, 55 °C; agitation speed, 300 rpm; aeration, 0.5 vvm; the initial pH of the culture medium 8.5; inoculum size, 1%. The culture broth was centrifuged and cell free supernatant was used for further purification.

2.2. Lipase assay

Lipase activity was measured with a pH-stat method. One enzyme unit was defined as enzyme amount that released 1 μM fatty acid from tributyrin per min at 60 °C and pH 8.5.

Lipase activity was also estimated using a spectrophotometric assay [11] with *p*-nitrophenyl palmitate (*p*NPP) as a substrate. One enzymatic unit was the amount of enzyme liberating one μM *p*NPP min^{-1} under the above conditions. The molar absorption coefficient of *p*NPP was determined to be 1.46×10^5 cm^2 M^{-1}.

2.3. Purification procedure

The purification method consisted of sequential ultrafiltration, Sephadex G-200 gel-chromatography and ion-exchange chromatography on DEAE Cellulose was applied.

Step 1: Culture supernatant (728 ml) was obtained by centrifugation of the culture broth at 4000 × *g* for 30 min. Ultrafiltration was performed using a Millipore system (membrane pore size of 30 000 Da).

Step 2: The concentrate was chromatographed on Sephadex G-200 column (2.6 cm × 55 cm) equilibrated with 0.01 M Tris–HCl buffer, pH 8.5 and then the enzyme was eluted with the same buffer. The flow rate was adjusted to 24.8 ml h^{-1}, the fraction volume of 3.5 ml was collected.

Step 3: The enzyme containing fractions were applied to a DEAE Servacell column (1.6 cm × 28 cm) previously equilibrated with the above buffer. The column was washed with two bed volumes of the same buffer and bound proteins were eluted with 60 ml linear NaCl gradient (0–1 M) in the same buffer. The flow rate was adjusted to 15 ml h^{-1}, the fraction volume of 3.5 ml. The active fractions were pooled and used for polyacrylamide gel electrophoresis and enzyme characterization.

2.4. Polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) was performed according to Laemmli [12]. Boiling time in sample buffer containing 7.5% 2-mercaptoethanol was 10 min and gels were stained with Coomassie Brilliant Blue G-250.

2.5. Kinetic study

The effect of substrate concentration (*p*NP palmitate) on the reaction rate was assayed for 10 min at pH 8.5 by using spectrophotometric method. The Michaelis Menten constant (K_m) and the maximum velocity for the reaction (V_{max}) were calculated by Lineweaver–Burke plot.

2.6. Substrate specificity

Substrate specificity of the enzyme was determined at 60 °C and pH 8.5 by pH-stat method using 1% emulsions of mono- and triglycerides. Substrate specificity towards *p*NP esters was determined colourimetrically.

2.7. Immobilization

The following compounds were used as carriers for ionic binding: Acrylex P30, Amberlite IRC 50 and IR 120, *p*-aminobenzyl Cellulose (Biochemical, Poole, UK), Dowex (Fluka), DEAE Cellulose (Reanal, Budapest, Hungary). Chitin (Protan, Drammen Norway) was used for physical adsorption. Preliminary treatment of the carriers was carried out according to the methods described by Kusano et al. [13]. One ml of ultrafiltrate was incubated overnight with 100 mg of the corresponding carrier and then the complex was thoroughly washed with 0.01 M Tris–HCl (pH 8.5) buffer. Ten mg fractions of bound lipase were incubated at 60 °C and activity yield and immobilized yield were determined according to:

$$\text{activity yield (\%)} = \frac{C}{A} \times 100$$

$$\text{immobilized yield (\%)} = \frac{A - B}{A} \times 100,$$

Table 1
Purification of lipase from *B. stearothermophilus* strain MC 7

Purification step	Volume (ml)	Total protein (g)	Total activity[a] (U)	Specific activity ($U\,g^{-1}$)	Purification factor	Yield (%)
Crude culture supernatant	728	2641.9	1638	0.62	1	100
Ultrafiltrate	98	264.6	1228.5	0.98	1.56	75
Sephadex G-200	360	14.4	982.5	10.21	16.47	60
DEAE-cellulose	54.6	4.56	167.1	11.94	19.25	10.2

[a] Activity was measured with pNP palmitate as a substrate.

where *A* is the amount of *p*NPP released by the enzyme added to the immobilization solution, *B* the amount of *p*NPP produced by the residual enzyme in the supernatant and *C* the amount of *p*NPP produced by the immobilized enzyme.

3. Results and discussion

3.1. Purification

The extracellular lipase by the thermophilic bacterium *B. stearothermophilus* MC 7 was purified employing a three-step procedure. The purification profile is summarized in Table 1. The use of dissociating agent Triton X-100 was critical to the success of the purification procedure at all stages. Lipolytic activity coeluted from Sephadex G-200 as high molecular weight proteins, suggesting that natively it exists in aggregated form. Forming of aggregates between either pure lipase or lipase and lipophilic molecules such as lipopolysaccharides, fatty acids, and glycerides, is not common for *Bacillus* lipase [8]. A strong tendency to form aggregates was established for *Bacillus subtilis* 168 [14]. The aggregation has been observed for lipases from other microorganisms like *Pseudomonas* [15] and *Serratia marcescens* [16]. Lipase eluted in 0.57 M NaCl during the DEAE Cellulose ion-exchange step. Coomassie Brilliant Blue staining revealed the presence of a single protein of a molecular weight of 62 500 Da (Fig. 1). Most *Bacillus* lipases have low molecular weight of about 20 000 Da [17]. Like lipases from *Bacillus thermocatenulatus* BTL2 [18], *Bacillus* sp. 398 [19] and *Bacillus* sp. J33 [8], the enzyme from *B. stearothermophilus* MC 7 is a large lipase. The enzyme was purified 19.25-fold with 10.2% recovery and a specific activity of about 12 U (mg protein)$^{-1}$. Generally, the yield in lipase purification procedure is comparatively low—between 2 and 20%. The responsibility of aggregation-related problems for the low yield of purification was speculated by other authors [14].

3.2. Effect of pH and temperature

Like most lipases, *B. stearothermophilus* MC 7 lipase had pH optimum within the range of 7.5–9.0 (correspondingly 6.9–7.9 at 60 °C) and was stable at alkaline pH range 7.0–11.0 (correspondingly 6.5–10.9 at 60 °C) as can be seen from Fig. 2.

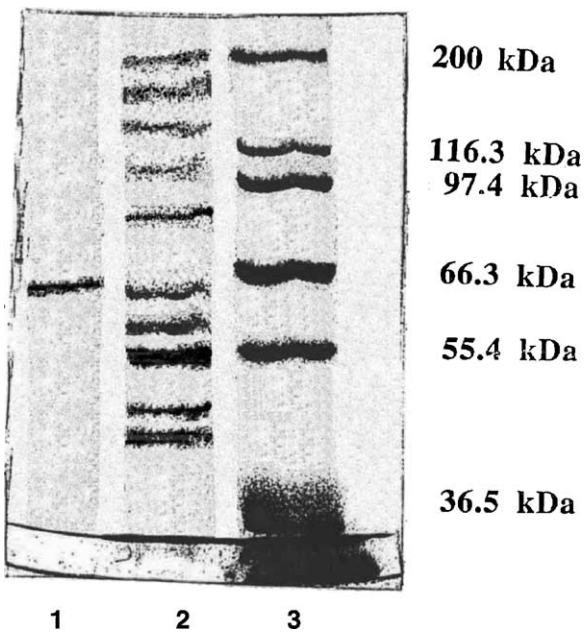

Fig. 1. SDS-PAGE analysis of purified lipase: (1) purified lipase; (2) culture liquid; (3) marker proteins—lactate dehydrogenase 36 500 Da; glutamic dehydrogenase 55 400 Da; bovine serum albumin 66 300 Da; phosphorilase B 97 400 Da; beta-galactosidase 116 300 Da; myosin 200 000 Da.

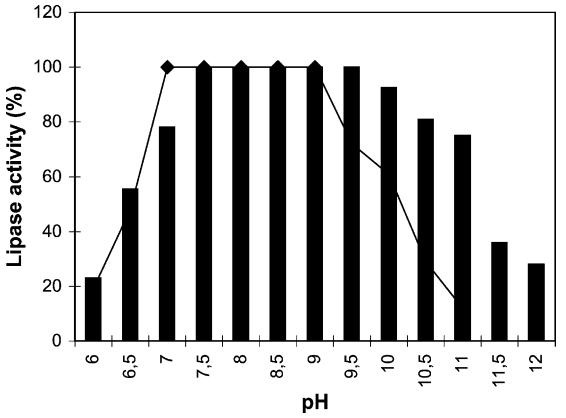

Fig. 2. Effect of pH on the activity and stability of *B. stearothermophilus* MC 7 lipase. pNP palmitate was used as a substrate. The following 50 mM buffers were used: phosphate (pH 5.0–8.5), Tris–glycine (pH 8.5–10.5), and Tris–NaON (pH 10.5–12) adjusted at room temperature. Next correspondence in pH values at 20 and 60 °C was established: 5.0–4.4; 5.5–5.0; 6.0–5.6; 6.5–6.1; 7.0–6.5; 7.5–6.9; 8.0–7.3; 8.5–7.5; 9.0–7.9, 9.5–8.5; 10–9.4; 10.5–10.4; 11.0–10.9; 11.5–11.4; 12.0–11.8. Activity (◆) was measured at different pH values and 60 °C with colourimetric method. pH stability (▮) was established after pre-incubation of lipase solution at different pH values during 30 min at 60 °C. Residual activity is expressed as percentage of original activity at pH 8.5, 60 °C.

The enzyme was very active between 55 and 90 °C with an optimal temperature of 75–80 °C (Fig. 3). Heating at 70 °C for 30 min half-inactivated pure enzyme, while crude lipase had half life 3 h at 70 °C. The half life of *B. stearothermophilus* MC 7 lipase is longer than those reported for other thermostable alkaline lipases: by Kim et al. ($t_{1/2}$ 30 min at 65 °C) [19], Schmidt-Dannert et al. ($t_{1/2}$ 30 min at 60 °C) [1] and Kim et al. ($t_{1/2}$ 30 min at 62 °C) [20], but lower than reported by Wang et al. ($t_{1/2}$ 8 h at 75 °C) [3]. This thermostability refers the enzyme between the most stable alkaline lipases and is not in agreement with suggested by Lee et al. [7] speculation that small and compact proteins may confer a higher thermostability than the bulky proteins. Significant increasing

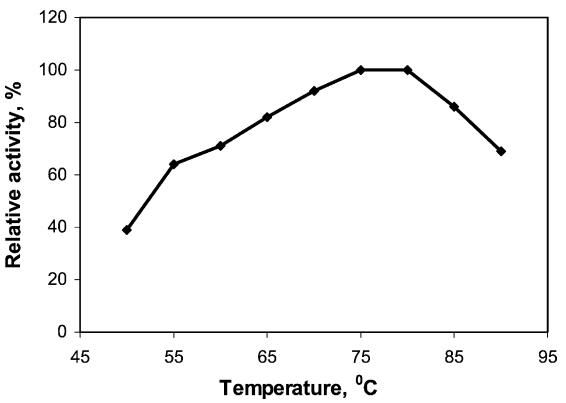

Fig. 3. Effect of temperature on the activity of *B. stearothermophilus* MC 7 lipase. Activity was measured at pH 8.5 by using pH-stat method for 10 min.

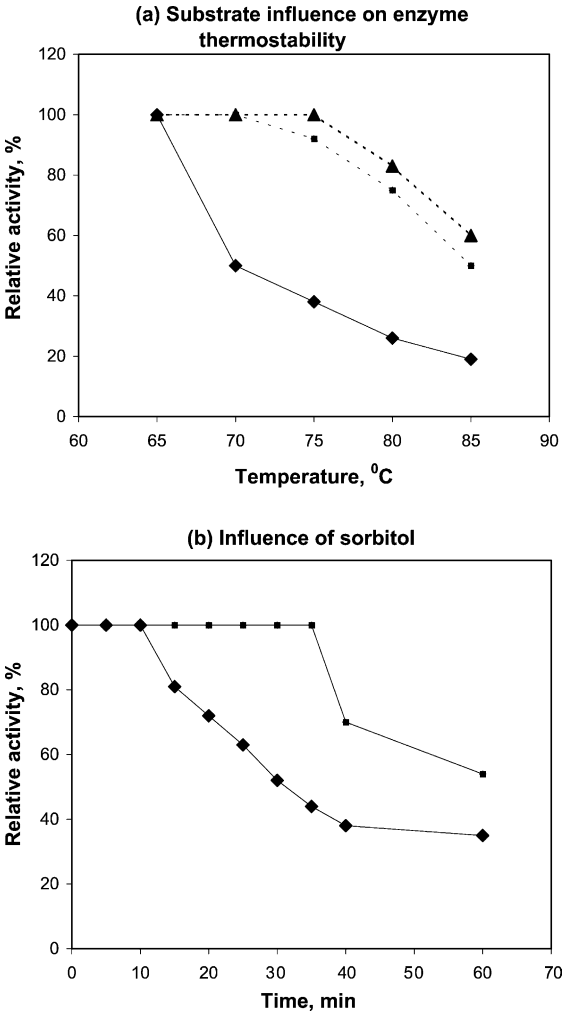

Fig. 4. Influence of additives on enzyme thermostability: (a) substrates—1% tributyrin (▲), 1% olive oil (■) and without substrate (◆); (b) presence of 10% sorbitol (■). Lipase solution was incubated for 30 min at different temperatures. Residual activity was measured by pH-stat method at 60 °C, pH 8.5.

of thermostability was established in presence of substrates tributyrin and olive oil (Fig. 4a) that has valuable practical importance. Sorbitol was recorded to be an effective additive enhancing enzyme thermostability (Fig. 4b). Similar effect was reported for the lipase from *Bacillus* sp. J33 [8]. The authors explained the influence of polyhydric alcohols by hindered denaturation due to dehydration in organic media.

K_m and V_{max} values were determined as a function of *p*NPP concentration to be 0.33 mM and 188 μM min^{-1} mg^{-1}.

3.3. Effect of different ions, inhibitors and organic solvents

B. stearothermophilus MC 7 lipase was inhibited by divalent ions of heavy metals, entirely by Cu^{2+} and strongly by Fe^{2+} and Zn^{2+} (Fig. 5a). Two possible mechanisms of ion action were suggested by Lee and Rhee [21]: (1) direct inhibition of the catalytic site, like many other enzymes; (2) specific for lipases formation of complexes between metal ions and ionized fatty acids, changing their solubility and behavior at

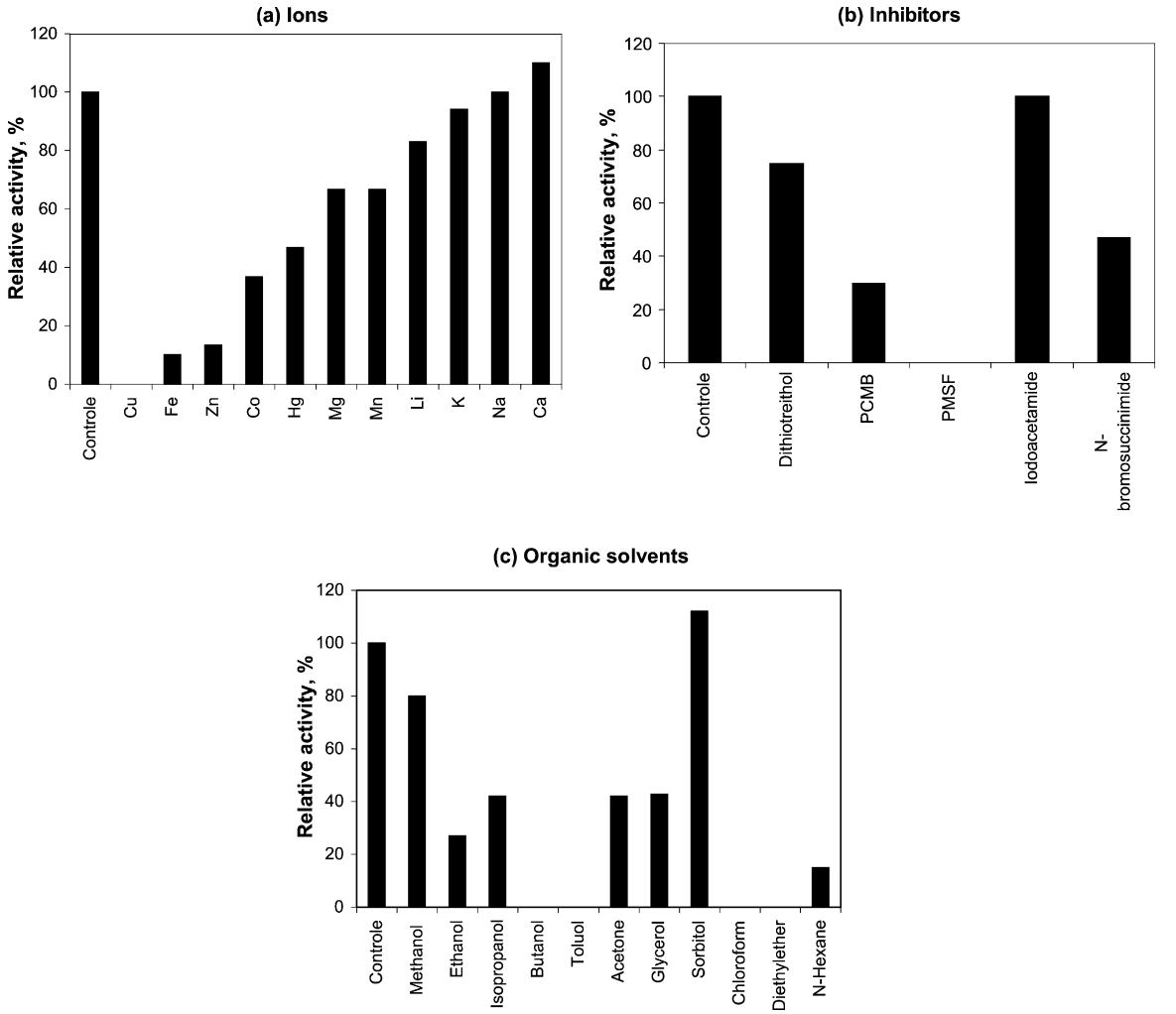

Fig. 5. Effect of some ions (a), inhibitors (b) and organic solvents (c) on lipase activity. The enzyme was incubated in Tris–HCl buffer, pH 8.5 containing different ions, inhibitors or organic solvents for 30 min at 70 °C. The final concentration of the ions and inhibitors was 5 mM, of the organic solvents 50%. Activity was then determined with colourimetric method at pH 8.5, 60 °C.

the interface. Like the most other lipases, the catalytic activity was slightly enhanced to about 110% in the presence of Ca^{2+}. The positive effect is generally accepted as a result of forming of insoluble Ca-salts of fatty acids released in the hydrolysis and thus product inhibition is avoided.

The activity was strongly affected by thiol inhibitor PCMB (Fig. 5b) that indicates an important role of SH-groups in the catalytic mechanism. Complete inhibition by PMSF, a serine inhibitor, probably was caused by modification of an essential serine residue in the active site.

B. stearothermophilus MC 7 lipase exhibited good tolerance to some organic solvents and polyhydric alcohols, however, full inhibition by butanol, toluol, chloroform, and diethylether was established (Fig. 5c).

3.4. Substrate specificity

The enzyme was able to hydrolyze both soluble and insoluble emulsified substrates. It demonstrated wide substrate specificity towards of substrates from C_4 to C_{18} (Table 2) with highest affinity to tributyrin. Likely lipase from *Pseudomonas aeruginosa* MB 5001 [15], that from *B. stearothermophilus* MC 7 hydrolyzed short-chain fatty acids more readily than those containing long-chain fatty acids. Triacylglycerols were preferred substrates while monoacylglycerols were hydrolyzed very slowly. The speed of hydrolysis for saturated (tristearin, C_{18}) and unsaturated (triolein, $C_{18:1}$) fatty acids was similar. Despite of some esterase activity expressed towards Tweens and triacetin (substrates characterizing esterase activity according to Plou et al. [22]), it didn't present typical for esterases substrate specificity pattern, i.e. activity only on soluble esters containing short fatty acids. Its ability to hydrolyze olive oil, insoluble triesters and PNPE of long-chain fatty acids determined the enzyme as a lipase, expressing some esterase activity as well. Similar conclusion for a responsibility of a single enzyme for both lipase and esterase activity was reached by Gilbert et al. [23].

Table 2
Substrate specificity of *B. stearothermophilus* MC 7 lipase

Substrate	Relative activity (%)
Triacylglycerols[a]	
Triacetin C_2	27
Tributyrin C_4	100
Tricaprin C_{10}	68
Trilaurin C_{12}	34
Tripalmitin C_{16}	34
Tristearin C_{18}	42
Triolein $C_{18:1}$	47
Tween 80	34
Olive oil	12
Monoacylglycerols[a]	
DL-α-Palmitin	12
DL-α-Stearin	7.8
p-Nitrophenylesters[b]	
p-Nitrophenyl acetate	15.2
p-Nitrophenyl palmitate	100

[a] Activity toward olive oil was accepted as 100%.
[b] Activity toward pNPP was accepted as 100%.

The results from lipase immobilization to the various carriers are summarized in Table 3. The most suitable exchanger for immobilization was DEAE Cellulose. The only reported in the literature immobilized thermophilic lipase was efficiently attached to Phenyl Sepharose [4]. The thermostability of the enzyme from *B. stearothermophilus* MC 7 was not significantly affected by immobilization process, its half life at 70 °C

Table 3
Immobilization of *B. stearothermophilus* MC 7 lipase

Carrier	Enzyme activity ($U\,g^{-1}$)		Immobilized activity (C)	Activity yield (%) ($C/A \times 100$)	Immobilized yield (%) ($(A - B)/A \times 100$)
	Added (A)	Unbound (B)			
Acrylex P30	18	1.99	0.69	3.8	88.9
Amberlite IRC 50	18	0	12.1	67.2	100
Amberlite IR 120	18	2.74	0.69	3.8	84.7
AB cellulose	18	2.47	2.1	11.7	86.3
Dowex 1	18	3.57	0	0	80.2
DEAE-cellulose	18	0	15.5	86.1	100
Chitin	18	2.3	11.3	15.92	87.2

was 37 min. The effectiveness of immobilization process is an important advantage for possible industrial application of this enzyme in continuous processes.

4. Conclusion

The results obtained in this study show that *B. stearothermophilus* MC 7 lipase is, to our knowledge, one of the most stable yet reported. This result renders the enzyme very attractive for biotechnological application. Based on the wide substrate specificity at elevated temperatures it can be inferred its use for different purposes in the fat and oil industry.

References

[1] C. Schmidt-Dannert, H. Sztajer, W. Stöcklein, U. Menge, R.D. Schmid, Biochim. Biophys. Acta 1214 (1994) 43.
[2] P.H. Janssen, C.R. Monk, H.W. Morgan, FEMS Microbiol. Lett. 120 (1994) 195.
[3] Y. Wang, K.C. Srivastava, G.J. Shen, H.Y. Wang, J. Ferment. Bioeng. 79 (1995) 433.
[4] P. Becker, I. Abu-Reesh, S. Markossian, G. Antranikian, H. Märkl, Appl. Microbiol. Biotechnol. 48 (1997) 184.
[5] L. Fakhreddine, A. Kademi, N. Aït-Abdelkader, J.C. Baratti, Biotechnol. Lett. 20 (1998) 879.
[6] Bradoo, R.K. Saxena, R. Gupta, World J. Microbiol. Biotechnol. 15 (1999) 87.
[7] D.-W. Lee, Y.-S. Koh, K.-J. Kim, B.-C. Kim, H.-J. Choi, D.-S. Kim, M.T. Suhartono, Y.-R. Pyun, FEMS Microbiol. Lett. 179 (1999) 393.
[8] N. Nawani, J. Kaur, Mol. Cell. Biochem. 206 (2000) 91.
[9] E. Emanuilova, M. Kambourova, M. Dekovska, R. Manolov, FEMS Microbiol. Lett. 108 (1993) 247.
[10] M. Kambourova, E. Emanuilova, P. Dimitrov, Folia Microbiol. 41 (1996) 146.
[11] U.K. Winkler, M. Stuckmann, J. Bacteriol. 138 (1979) 663.
[12] U.K. Laemmli, Nature 227 (1970) 680.
[13] S. Kusano, T. Shiraishi, S.-I. Takahashi, D. Fujimoto, Y. Sakano, J. Ferment. Bioeng. 68 (1989) 233.
[14] E. Lesuisse, K. Schanck, C. Colson, Eur. J. Biochem. 216 (1993) 155.
[15] M. Chartain, L. Katz, C. Marcin, M. Thien, S. Smith, E. Fisher, K. Goklen, P. Salmon, T. Brix, K. Price, R. Greasham, Enzyme Microb. Technol. 15 (1993) 575.
[16] H. Matsumae, T. Shibatani, J. Ferment. Bioeng. 77 (1994) 152.
[17] A. Sugihara, T. Tani, Y. Tominaga, J. Biochem. 109 (1991) 211.
[18] C. Schmidt-Dannert, M.L. Rua, R.D. Schmid, in: B. Rubin, E.A. Dennis (Eds.), Methods in Enzymology, vol. 284, Academic Press, New York, USA, 1997, p. 194.
[19] H. Kim, M. Sung, H. Kim, T. Oh, Biosci. Biotech. Biochem. 58 (1994) 961.
[20] H. Kim, M. Sung, H. Kim, T. Oh, Biosci. Biotechnol. Biochem. 62 (1998) 66.
[21] S.Y. Lee, J.S. Rhee, Enzyme Microb. Technol. 15 (1993) 617.
[22] F.J. Plou, M. Ferrer, O.M. Nuero, M.V. Calvo, M. Alcalde, F. Reyes, A. Ballesteros, Biotechnol. Tech. 12 (1998) 183.
[23] E.J. Gilbert, A. Cornish, C.W. Jones, J. Gen. Microbiol. 137 (1991) 2223.