Appl Biochem Biotechnol (2011) 163:304–312
DOI 10.1007/s12010-010-9039-4

Immobilization of a Recombinant Esterase from *Lactobacillus plantarum* on Polypropylene Accurel MP1000

Deise Juliana Kolling · Willian Alexandre Suguino · Fábio Cristiano Angonesi Brod · Ana Carolina Maisonnave Arisi

Received: 18 April 2010 / Accepted: 5 July 2010 /
Published online: 23 July 2010

Abstract A recombinant esterase from *Lactobacillus plantarum* was immobilized on hydrophobic support polypropylene Accurel MP1000 by adsorption. Adsorption efficiency was 83%, and the immobilized protein was 12.4 mg/g of support. Esterase activity was determined using *p*-nitrophenyl butyrate as substrate, and highest activities were observed at 50 °C for immobilized enzyme and 30 °C for free enzyme extract. Concerning thermal stability, after enzyme incubation at 80 °C for 30 min, immobilized and free enzyme retained 91% and 56% of initial activity, respectively. Immobilized enzyme presented lower V_{max} and higher K_m than free enzyme. Protein was not released from the support, and esterase activity increased after 3 cycles of reuse.

Keywords Esterase · *Lactobacillus plantarum* · Immobilization · Accurel MP1000 · Recombinant protein · Lactic acid bacteria

Introduction

Enzyme immobilization is an important technique which ensures recycling of the biocatalyst, permits easy product separation, and improves performance of the enzyme [1–3]. Kinetic behavior of immobilized enzyme is different from free enzyme due to conformational changes, steric, partition, microenvironmental and diffusion effects. Commonly used immobilization methods include simple adsorption of the enzyme to the surface of a solid support, covalent binding of the enzyme to a solid support, encapsulation, and entrapment. Adsorption is the most commonly used method because of its simplicity [2, 4]. Adsorption of a protein onto a solid surface is a widely used method and porous polypropylene Accurel has been used to immobilize different hydrolases [1, 5–7].

D. J. Kolling · W. A. Suguino · F. C. Angonesi Brod · A. C. M. Arisi (✉)
Departamento de Ciência e Tecnologia de Alimentos, Universidade Federal de Santa Catarina, Rod. Admar Gonzaga, 1346, 88034-001 Florianópolis, Brazil
e-mail: arisi@cca.ufsc.br

Esterases belong to the general class of carboxylic ester hydrolases (EC 3.1.1). They are serine hydrolases capable of synthesizing or hydrolyzing esters depending on the physicochemical conditions. Esterases can be used in media containing low water content, where they catalyze reactions such as esterification or transesterification. These reactions led to several potential applications as synthesis of flavor esters for food industry [8] and wine industry [9], the resolution of racemic mixtures by transesterification, or enantioselective hydrolysis of esters for obtaining optically pure compounds [10].

Esterase enzymes of lactic acid bacteria represent a diverse group of enzymes that may have multiple activities depending on the substrate available and on the environment in which the enzyme is operating [9]. *Lactobacillus plantarum* is a well-known lactic acid bacteria being often associated with meat and dairy fermented products [11]. Information about lactic acid bacteria as a source of useful enzymes is scarce, especially concerning esterases from *Lactobacillus* spp. Reports describing cloning, expression, and characterization of six esterases from lactic acid bacteria have been published, EstA (AF157601) from *Lactococcus lactis* subsp. lactis (B1014), EstB (AF494421) and EstC (AF506279) from *Lactobacillus casei* LILI, EstI (AY251019) from *L. casei* CL96, EstA (AF136284) from *Lactobacillus helveticus* CNRZ32, and EstLpl (AL935263) from *L. plantarum* ATCC8014 [9, 12–16].

Recently, an esterase from *L. plantarum* was cloned, purified by immobilized metal-ion affinity chromatography, and characterized [16]. It presented an apparent molecular mass of about 38 kDa, optimal catalytic activity at pH 6.0 and 40 °C, and preference for substrates containing short-chain fatty acids. The aim of the present study was to immobilize the recombinant esterase from *L. plantarum* [16] on polypropylene Accurel MP1000. Catalytic activities of the immobilized and free enzymes were compared, and thermal stability and reuse of the immobilized enzyme were tested.

Material and Methods

Bacterial Strain, Plasmid, Support, and Chemicals

Escherichia coli strain BL21 (DE3) pLysS containing the plasmid *pET14b-Est_Lpl* [16] was used for protein expression of *L. plantarum* esterase. The *p*-nitrophenyl butyrate ($pNPC_4$), isopropyl-L-thio-β-D-galactopyranoside (IPTG), phenylmethylsulfonyl fluoride (PMSF), and other chemicals were purchased from Sigma. The support used for enzyme immobilization was microporous polypropylene (Accurel MP1000) supplied for Accurel® Systems.

Recombinant *L. plantarum* Esterase

E. coli BL21 (DE3) pLysS harboring the *pET14b-Est_Lpl* vector were inoculated in 10 mL LB broth supplemented with 100 μg/ml ampicillin and 50 μg/ml chloramphenicol. Overnight cultures were transferred to 250 ml of the same medium and cultivated at 37 °C until OD_{600} of 0.8. IPTG was added to a final concentration of 1 mmol/l, incubated at 15 °C for 18 h. Cells were harvested by centrifugation (3,000×*g*, 15 min, 4 °C), and pellet was kept at −20 °C. Pellet was resuspended in 2 mL of 50 mM phosphate buffer pH 8.0 containing 40 μg/ml of PMSF, disrupted by gentle sonication (7 cycles, 20 s) on ice, and centrifuged (10,000×*g*, 20 min, 4 °C) twice in order to obtain the esterase extract. Extract containing the recombinant esterase was filtered on Millipore 0.22 μm and kept at 4 °C

until immobilization. Protein concentration was determined using a Bio-Rad protein assay kit with bovine serum albumin as standard.

Esterase Immobilization onto Polypropylene

Recombinant esterase from *L. plantarum* was immobilized on Accurel MP1000 by adsorption. One hundred milligrams of Accurel was placed in 250 μl of ethanol. Esterase extract was diluted in 3 ml phosphate buffer (24 mM, pH 7.0) to final protein concentration of 1 mg/ml, added to 100 mg of support previously treated with ethanol, incubated at 24 °C for 18 h under agitation (120 rpm), and then filtered and washed with water at 4 °C. After incubation at 30 °C for 6 h, dried immobilized esterase was kept at 4 °C until enzyme activity assay. Aliquots from initial and final solutions were collected, and protein content and enzyme activity were quantified in order to determine immobilization yield.

Esterase Activity

A stock solution of 100 mmol/l of *p*-nitrophenyl butyrate ($pNPC_4$) was prepared in isopropanol and mixed with 50 mM phosphate buffer (pH 6.0) in order to obtain the reaction buffer containing 1 mmol/l $pNPC_4$. To initiate the reaction, 30 mg of support containing the immobilized esterase was mixed with 3 ml of reaction buffer at 40 °C for 30 min under agitation (100 rpm). In order to stop the reaction, 750 μl of 100 mmol/l Na_2CO_3 was added [8], and immobilized enzyme was separated by centrifugation (9.3×*g*, 5 min, 24 °C). Enzyme activity was determined by quantification of *p*-nitrophenol at 348 nm in spectrophotometer (HITACHI U2910). Blanks consisted of the reaction buffer containing the Accurel support processed under the same conditions. Molar extinction coefficient for *p*-nitrophenol was experimentally determined as 5,487.25 $M^{-1}cm^{-1}$. In order to investigate temperature effect, enzyme activities of free and immobilized enzyme were carried out at 30, 40, 50, and 60 °C as described above.

Kinetic Parameters

Esterase activity was measured as a function of different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, and 3 mM) of $pNPC_4$. Michaelis–Menten substrate affinity constant (K_m), maximum velocity (V_{max}), and the turnover number (k_{cat}) were calculated using GraphPad Prism 5.01 software.

Thermal Stability

Thermal stability was performed by incubating 30 mg of support containing immobilized enzyme in 1 ml of 50 mM phosphate buffer, pH 6.0, for 10, 20, 30, and 60 min. After incubation, 1.85 ml of 50 mM phosphate buffer, pH 6.0, were added, and solution was incubated at 40 °C for 10 min. For enzyme activity, 150 μl of 20 mM $pNPC_4$ were added, and activity assay was carried out as described above.

Operational Stability

Efficiency reusing of immobilized enzyme was determined as described above using 50 mg of support containing immobilized esterase and 5 ml of reaction buffer. After enzymatic assay, immobilized esterase was collected by filtration and washed with ultrapure water in

order to remove reactants from activity assay. Then, support containing immobilized enzyme was resuspended in fresh reaction buffer in order to start a new run. A total of six runs were carried out for testing operational stability of immobilized esterase.

Desorption Assay

A desorption assay was performed in order to detect protein desorption from support. In this way, 30 mg of support containing immobilized esterase were incubated in 2 ml of 50 mM phosphate buffer, pH 6.0, at 40 °C on a shaker (100 rpm). Aliquots were collected every 30 min, and protein content was determined using a Bio-Rad protein assay kit with bovine serum albumin as standard.

Results and Discussion

L. plantarum recombinant esterase was previously cloned, sequenced, and expressed in *E. coli* BL21 (DE3) pLysS harboring the plasmid *pET14b-Est_Lpl* [16]. The same *E. coli* strain was used for expression of *L. plantarum* recombinant esterase in order to immobilize the enzyme. Transformed cells were grown in the presence of IPTG 1 mM for 18 h at 15 °C. After centrifugation, cell pellet was resuspended in phosphate buffer and disrupted by sonication. Obtained supernatant was centrifuged twice and filtered. Soluble esterase extract was then immobilized onto polypropylene Accurel MP1000. Immobilization was carried out by adsorption, which usually causes a slight decrease of enzyme activity. Immobilization in polypropylene Accurel MP1000 was successfully performed, immobilization efficiency was of 83±4% (based on esterase activity), and amount of adsorbed enzyme was of 12.4±5.4 mg/g of support. This result is higher than previously reported, a lipase extract from *Geobacillus thermoleovarans* immobilized in polypropylene resulted in 8.7 mg/g of adsorbed protein per support [7], and a lipase from *Candida rugosa* immobilized in polypropylene resulted in 8.1 mg/g of adsorbed protein per support [17]. A recombinant thermostable esterase (Pf2001) from *Pyrococcus furiosus* immobilized in polypropylene Accurel MP-1000 resulted in 35.5 mg/g of adsorbed protein per support [6]. According to Almeida et al. (2008), there is a large variation of results depending on the adsorption system, where enzyme characteristics, support superficial area, and particle and pore size seem to play an important role in the adsorption process.

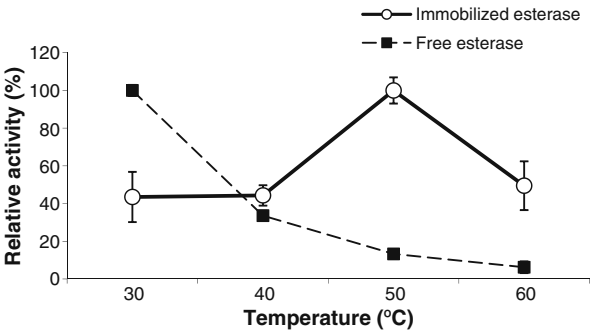

Fig. 1 Effect of temperature on free and immobilized *L. plantarum* esterases. Enzyme activity was measured using $pNPC_4$ at pH 6.0. Results are mean±SE of four replications

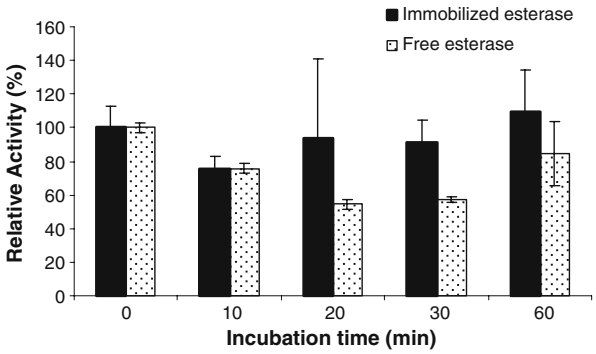

Fig. 2 Thermal stability of free and immobilized *L. plantarum* esterases. Enzyme activity was measured using $pNPC_4$ at pH 6.0 and 40 °C after incubation at 80 °C for 10, 20, 30, and 60 min. Results are mean±SE of four replications

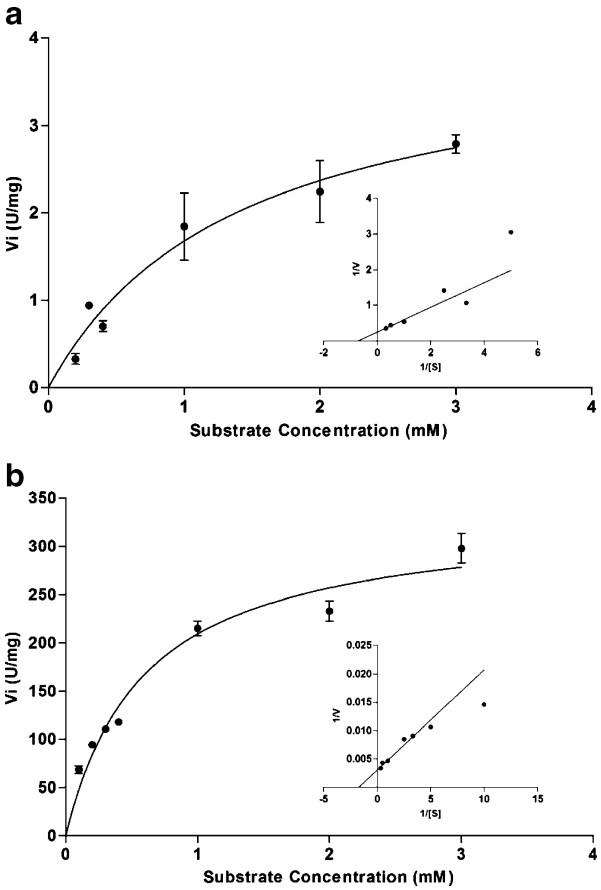

Fig. 3 Michaelis–Menten plots of **a** immobilized esterase and **b** free esterase. Kinetic data were measured using $pNPC_4$ as substrate in 50 mM phosphate buffer pH 6.0 at 40 °C. *Inset graphs* show the Lineweaver–Burk plot of the transformed data. Results are mean±SD of four replications

Table 1 Kinetic parameters of immobilized and free *L. plantarum* recombinant esterases.

Enzyme	Specific activity ($U mg^{-1}$)	V_{max} ($nmol min^{-1} mg^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} mM^{-1}$)
Immobilized	1.8±0.3	4.0±0.6	1.39±0.48	2.61×10^{-3}	1.87×10^{-3}
Free enzyme	215.1±7.6	333.1±15.1	0.59±0.07	2.16×10^{-1}	3.65×10^{-1}

Results are mean±SD of four independent experiments

Effect of Temperature on Activity and Stability of Free and Immobilized Enzyme

Effect of temperature on hydrolysis activity of free and immobilized enzyme was evaluated at 30, 40, 50, and 60 °C (Fig. 1). Free enzyme presented 100% activity at 30 °C and 33%, 13% and 6% activity at 40, 50, and 60 °C, respectively. Immobilized enzyme presented 100% activity at 50 °C and 40–50% activity at 30, 40, and 60 °C. It has been observed that immobilized enzyme showed a similar temperature behavior as already reported by other authors [18, 19]. Esterase activity of free and immobilized esterase extract were evaluated at 40 °C, once both presented 35% of highest activity at 40 °C. Other authors have shown that immobilized enzyme activity is higher than free enzyme activity at temperatures above 40 °C [18–22]. Recombinant esterase from *L. plantarum* purified by immobilized metal affinity chromatography presented highest hydrolysis activity for $pNPC_4$ at 40 °C [16]. Otherwise, free recombinant *L. plantarum* esterase extract used in this study presented highest activity at 30 °C (Fig. 1).

An important parameter has been thermal stability, largely investigated for industrial applications. The thermal stability was determined by the incubation of free and immobilized enzyme extracts at 80 °C for different time intervals. After the incubation at 40 °C for 10 min, esterase activity was measured at 40 °C (Fig. 2). After 30 min of incubation, immobilized enzyme presented higher residual activity (91%) than free enzyme (56%). This result was in agreement with other studies in which immobilized enzyme in hydrophobic supports presented higher thermal stability than free enzyme [2, 7, 23]. Immobilization could promote a restriction on the protein conformational motility, and it could cause high rigidity in enzyme structure, the prevention of unfolding and loss of catalytic activity [2, 7, 23]. The immobilization by adsorption seems to play an important role in the stabilization of the enzyme structure and resistance to denaturation [2].

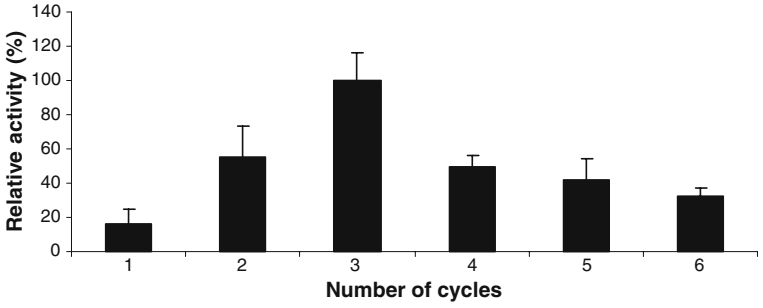

Fig. 4 Operational stability of immobilized *L. plantarum* esterase. Enzyme activity was measured using $pNPC_4$ at pH 6.0 and 40 °C. Results are mean±SE of four replications

Kinetic Parameters of Free and Immobilized Enzyme

Kinetic constants were determined using different concentrations of substrate $pNPC_4$ (Fig. 3). V_{max}, K_m, k_{cat}, and k_{cat}/K_m values were obtained from Michaelis–Menten plot, and the values for immobilized enzyme refer to milligram protein bound to the polymer (Table 1). V_{max} was lower for immobilized enzyme. After immobilization process, a decrease in reaction velocity is usually observed, as reported by other authors [19, 24, 25]. K_m value of immobilized enzyme was lower than value found for free enzyme, evidencing a decrease in substrate enzyme affinity after immobilization process (Table 1). Turnover number, k_{cat}, gives the number of substrate molecules converted in product by the active site for second, and ratio k_{cat}/K_m relates catalytic efficiency and substrate affinity. Evaluation of these parameters showed that immobilized enzyme presented values lower than those found for free enzyme. The low value found for k_{cat}/K_m of immobilized enzyme is related to its low catalytic efficiency. The advantage of the immobilized enzyme is the reutilization; otherwise, the free enzyme could not be reused.

Operational Stability of Immobilized Enzyme

Desorption assay was carried out in order to determine the loss of immobilized protein during hydrolysis reaction; until the third cycle, there was no loss of protein content, determined by Bradford method. Chemical nature of the support, immobilization process, and operational conditions strongly influence on the number of cycles for immobilized enzyme re-using. One of the main disadvantages of immobilization by adsorption is that enzyme presents loss of stability when reused, mainly in aqueous media [7]. In the present study, reutilization assay of the immobilized enzyme showed an atypical result until third cycle due to an increasing in enzyme activity (Fig. 4). However, for fourth to sixth cycles, a typical decreasing in catalytic activity was observed. Such decreasing is usually observed and was reported by other authors [7, 25–27].

Conclusion

Efficiency of adsorption process of recombinant esterase from *L. plantarum* in microporous polypropylene Accurel MP1000 was 83%. Immobilized enzyme presented highest activity at 50 °C. Immobilized recombinant esterase from *L. plantarum* presented thermal stability, retaining 91% of its activity after incubation at 80 °C for 30 min, and after 3 cycles of reuse, no protein desorption was detected. Moreover, immobilized enzyme remained active after 6 cycles of reuse. It is the first report describing the immobilization of a lactic acid bacteria esterase.

Acknowledgments This work was financially supported by CNPq process 552508/2007-1. DJK and FCAB were recipients of fellowships from CAPES, and WAS was a recipient of fellowship from CNPq. We would like to express our gratitude to Dr. Elisa Helena Siegel Moecke for fruitful discussion.

References

1. Sabbani, S., Hedenstrom, E., & Nordin, O. (2006). The enantioselectivity of *Candida rugosa* lipase is influenced by the particle size immobilising support material Accurel. *Journal of Molecular Catalysis. B, Enzymatic, 42*, 1–9.
2. Alloue, W., Destain, J., El Medjoub, T., Ghalfi, H., Kabran, P., & Thonart, P. (2008). Comparison of *Yarrowia lipolytica* lipase immobilization yield of entrapment, adsorption, and covalent bond techniques. *Applied Biochemistry and Biotechnology, 150*, 51–63.

3. Cunha, A. G., Fernandez-Lorente, G., Gutarra, M. L. E., Bevilaqua, J. V., Almeida, R. V., Paiva, L. M. C., et al. (2009). Separation and immobilization of lipase from *Penicillium simplicissimum* by selective adsorption on hydrophobic supports. *Applied Biochemistry and Biotechnology, 156*, 563–575.
4. Lumor, S. E., & Akoh, C. C. (2008). Esterification and hydrolytic activities of *Candida rugosa* lipase isoform 1 (LIP1) immobilized on celite 545, duolite A7, and sephadex G-25. *Journal of Agricultural and Food Chemistry, 56*(21), 10396–10398.
5. Gupta, N., Rathi, P., Singh, R., Goswami, V. K., & Gupta, R. (2005). Single-step purification of lipase from *Burkholderia multivorans* using polypropylene matrix. *Applied Microbiology and Biotechnology, 67*, 648–653.
6. Almeida, R. V., Branco, R. V., Peixoto, B., Lima, C. S., Alqueres, S. M. C., Martins, O. B., et al. (2008). Immobilization of a recombinant thermostable esterase (Pf2001) from *Pyrococcus furiosus* on microporous polypropylene: Isotherms, hyperactivation and purification. *Biochemical Engineering Journal, 39*, 531–537.
7. Sánchez-Otero, M. G., Valério-Alfaro, G., García-Galindo, H. S., & Oliart-Ros, R. M. (2008). Immobilization in the presence of Triton X-100: Modifications in activity and thermostability of *Geobacillus thermoleovorans* CCR11 lipase. *Journal of Industrial Microbiology and Biotechnology, 35*, 1687–1693.
8. Soliman, N. A., Knoll, M., Abdel-Fattah, Y. R., Schmid, R. D., & Lange, S. (2007). Molecular cloning and characterization of thermostable esterase and lipase from *Geobacillus thermoleovorans* YN isolated from desert soil in Egypt. *Process Biochemistry, 42*, 1090–1100.
9. Sumby, K. M., Grbin, P. R., & Jiranek, V. (2010). Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. *Food Chemistry, 121*, 1–16.
10. Torres, S., Baigori, M. D., Pandey, A., & Castro, G. R. (2008). Production and purification of a solvent-resistant esterase from Bacillus licheniformis S-86. *Applied Biochemistry and Biotechnology, 151*, 221–232.
11. Kleerebezem, M., Boekhorst, J., Van Kranenburg, R., Molenaar, D., Kuipers, O. P., Leer, R., et al. (2003). Complete genome sequence of Lactobacillus plantarum WCFS1. *Proceedings of the National Academy of Sciences of the United States of America, 100*(4), 1990–1995.
12. Fenster, K. M., Parkin, K. L., & Steele, J. L. (2000). Characterization of an arylesterase from Lactobacillus helveticus CNRZ32. *Journal of Applied Microbiology, 88*, 572–583.
13. Fernandez, L., Beerthuyzen, M. M., Brown, J., Siezen, R. J., Coolbear, T., Holland, R., et al. (2000). Cloning, characterization, controlled overexpression, and inactivation of the major tributyrin esterase gene of *Lactococcus lactis*. *Applied and Environmental Microbiology, 66*(4), 1360–1368.
14. Fenster, K. M., Parkin, K. L., & Steele, J. L. (2003). Intracellular esterase from Lactobacillus casei LILA: Nucleotide sequencing, purification, and characterization. *Journal of Dairy Science, 86*(4), 1118–1129.
15. Fenster, K. M., Parkin, K. L., & Steele, J. L. (2003). Nucleotide sequencing, purification, and biochemical properties of an arylesterase from Lactobacillus casei LILA. *Journal of Dairy Science, 86* (8), 2547–2557.
16. Brod, F. C. A., Vernal, J., Bertoldo, J. B., Terenzi, H., & Arisi, A. C. M. (2010). Cloning, expression, purification and characterization of a novel esterase from *Lactobacillus plantarum*. *Molecular Biotechnology, 44*, 242–249.
17. Minovska, V., Winkelhausen, E., & Kuzmanova, S. (2005). Lipase immobilized by different techniques on various support materials applied in oil hydrolysis. *Journal of the Serbian Chemical Society, 70*(4), 609–624.
18. Karra-Châabouni, M., Bouaziz, I., Boufi, S., Rego, A. M. B., & Gargouri, Y. (2008). Physical immobilization of *Rhizopus oryzae* lipase onto cellulose substrate: Activity and stability studies. *Colloids and Surfaces. B: Biointerfaces, 66*, 168–177.
19. Kumari, A., Mahapatra, P., Kumar, G. V., & Banerjee, R. (2008). Comparative study of thermostability and ester synthesis ability of free and immobilized lipases on cross linked silica gel. *Bioprocess and Biosystems Engineering, 31*, 291–298.
20. Huang, L., & Cheng, Z. (2008). Immobilization of lipase on chemically modified bimodal ceramic foams for olive oil hydrolysis. *Chemical Engineering Journal, 144*, 103–109.
21. Paula, A. V., Moreira, A. B. R., Braga, L. P., & Castro, H. F. (2008). Comparative performance of *Candida rugosa* lipase immobilized on polysiloxane polyvinyl alcohol hybrid support using different methodologies. *Quimica Nova, 31*, 35–40.
22. Deng, H.-T., Xu, Z.-K., Liu, Z.-M., & Ye, P. (2004). Adsorption immobilization of Candida rugosa lipases on polypropylene hollow fiber microfiltration membranes modified by hydrophobic polypeptides. *Enzyme and Microbial Technolgy, 35*, 437–443.
23. Nawani, N., Singh, R., & Kaur, J. (2006). Immobilization and stability studies of a lipase from thermophilic *Bacillus* sp: The effect of process parameters on immobilization of enzyme. *Electronic Journal of Biotechnology, 9*, 559–565.

24. Li, S., Hu, J., & Liu, B. (2004). Use of chemically modified PMMA microspheres for enzyme immobilization. *BioSystems, 77*, 25–32.
25. Ye, P., Xu, Z.-K., Che, Ai-Fu, Wu, J., & Seta, P. (2005). Chitosan-tethered poly(acrylonitrile-co-maleic acid hollow fiber membrane for lipase immobilization. *Biomaterials, 26*, 6394–6403.
26. Yagiz, F., Kazan, D., & Akin, A. N. (2007). Biodiesel production from waste oils by using lipase immobilized on hydrotalcite and zeolites. *Chemical Engineering Journal, 134*, 262–267.
27. Pahujani, S., Kanwar, S. S., Chauhan, G., & Gupta, R. (2008). Glutaraldehyde activation of polymer nylon-6 for lipase immobilization: Enzyme characteristics and stability. *Bioresource Technology, 99*, 2566–2570.