Appl Biochem Biotechnol (2010) 160:1146–1156
DOI 10.1007/s12010-009-8578-z

Screening of Food Grade Lipases to be Used in Esterification and Interesterification Reactions of Industrial Interest

Ariela Veloso de Paula · Gisele Fátima Morais Nunes · Josiane de Lourdes Silva · Heizir Ferreira de Castro · Júlio César dos Santos

Received: 15 December 2008 / Accepted: 18 February 2009 / Published online: 5 March 2009

Abstract Seven food grade commercially available lipases were immobilized by covalent binding on polysiloxane–polyvinyl alcohol (POS-PVA) hybrid composite and screened to mediate reactions of industrial interest. The synthesis of butyl butyrate and the interesterification of tripalmitin with triolein were chosen as model reactions. The highest esterification activity (240.63 μM/g min) was achieved by *Candida rugosa* lipase, while the highest interesterification yield (31%, in 72 h) was achieved by lipase from *Rhizopus oryzae*, with the production of about 15 mM of the triglycerides C_{50} and C_{52}. This lipase also showed a good performance in butyl butyrate synthesis, with an esterification activity of 171.14 μM/g min. The results demonstrated the feasibility of using lipases from *C. rugosa* for esterification and *R. oryzae* lipase for both esterification and interesterification reactions.

Keywords Food grade lipases · Interesterification · Esterification · Immobilization · Lipase

Introduction

Among a number of enzymes commercially available nowadays, lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) stand out as a versatile group of biocatalysts [1]. The reactions catalyzed by lipases are very important in many situations, such as in food, pharmaceutical, and chemical applications [1, 2]. Lipases can be used in hydrolysis of oils and fats to produce important fatty acids (FAs) as polyunsaturated FAs of omega-3 and omega-6 series [3]. In organic media, esterification reactions are important to produce flavor esters [4], monoglycerides [5], or other compounds of clinical interest [1]. Moreover, lipases can catalyze interesterification reactions, which can be used as a method to modify the physical

A. V. de Paula · G. F. M. Nunes · J. d. L. Silva · H. F. de Castro · J. C. dos Santos (✉)
Engineering School of Lorena, University of São Paulo, P.O. Box 116,
Lorena 12602-810 São Paulo, Brazil
e-mail: jsant200@yahoo.com.br

and chemical properties of fats and oils, a basic process for the production of the "structured lipids."

Although numerous reactions have been performed using lipases, it is still a challenge to identify the most suitable biocatalyst and the best reaction conditions for an efficient application [6]. Key aspects of lipase-catalyzed reactions that should be considered are source and specificity of lipase, the role of water, the effect of an organic solvent, and immobilization of the enzyme [6]. Of these, immobilization plays an import role, since this procedure isolates the biocatalyst from the reaction product and allows its reuse and thus reduce the overall process costs [5]. In addition, to the ease of handling, immobilized enzymes are well suited for use in continuous packed- or fluidized-bed reactors.

There are many methods available for biocatalyst immobilization that span from binding on prefabricated carrier materials to incorporation into in situ prepared carriers. Recently, the potential of using a hybrid matrix of polysiloxane–polyvinyl alcohol (POS-PVA) obtained by sol-gel process for lipase immobilization has been demonstrated in our laboratory for several lipases sources [7, 8]. This support was used to immobilize seven food grade lipases allowing their safe use in products for human feeding. The resulted immobilized derivatives were applied in the esterification of butanol with butyric acid and interesterification of triolein with tripalmitin; these reactions were chosen as study models. The objective of this work was to screen food grade commercially available lipases to be used in esterification and interesterification reactions, aiming their applications in oil and fat modifications.

Materials and Methods

Materials

Commercial lipases in a crude form were used in this study without further purification with an emphasis on practical application: *Aspergillus niger* (Lipase A) and *Mucor javanicus* (Lipase M) from Amano Enzyme Inc. (Nagoya, Japan); *Rhizopus oryzae* (Lipase L036P), *Candida rugosa* (Lipomod™ 34P), and *Penicillium roqueforti* (Lipomod™ 338P) from Biocatalysts (Cardiff, England); and *R. oryzae* (Piccantase® R8000) and *Rhizomucor miehei* (Piccantase® A) from DSM Food Specialties (Delft, The Netherlands). Tetraethoxysilane was acquired from Aldrich Chemical Co. (Milwaukee, WI, USA). Hydrochloric acid (minimum 36%), ethanol (minimum 95%), and polyethylene glycol (PEG 1500 g/mol) were supplied by Synth (acquired from Hipperquímica, Santo André, São Paulo, Brazil); PVA (MW 88,000) was supplied by Sigma-Aldrich (Sigma Aldrich Chemical Company, St. Louis, MO, USA). All other chemicals were of analytical grade.

Substrates

Commercial olive oil (low acidity, purchased in a local market) was used to determine the hydrolytic activity. *n*-Butanol (99%) and butyric acid, both purchased from Merck KGA (Darmstadt, Germany), were used as starting materials for esterification reactions. Triolein and tripalmitin, both purchased from Sigma-Aldrich (Sigma Aldrich Chemical Company, St. Louis, MO, USA), were used as substrate in the interesterification reactions. Substrates and solvent for esterification reactions were dehydrated with 0.32 cm molecular sieves (aluminum sodium silicate, type 13 X-BHD Chemicals, Toronto, Canada), previously activated in an oven at 350 °C for 6 h.

Support Synthesis and Activation Procedure

The POS-PVA hybrid composite was prepared by sol-gel technique according to the methodology described by Paula et al. [9]. The resulting POS-PVA particles were activated with sodium metaperiodate, as follows: The carrier was suspended in an aqueous solution of sodium metaperiodate (0.5 M) in the proportion of 1 g of carrier/10 mL solution. The mixture was maintained under agitation during 90 min, at 25 °C, in a dark place. After this, the carrier was filtered under vacuum and washed in abundance with distilled water and phosphate buffer pH 8.0 (0.1 M). After washing, the solid was maintained under vacuum for drying during 30 min, kept under 60 °C for 24 h, and used in the immobilization procedures.

Immobilization Procedure

Activated POS-PVA particles were soaked into hexane under stirring (100 rpm) for 1 h at 25 °C. Then, excess hexane was removed, and the particles were added with aqueous PEG 1500 solution (5 mg/mL) and lipase in the proportion of 200 mg of enzyme/1 g carrier/100 μL of PEG. Lipase support system was maintained in contact for 24 h at 4 °C. The immobilized lipase was recovered by filtration under vacuum, with subsequent washing with hexane. The coupling yield (η%) was calculated according to Eq. 1:

$$n(\%) = \frac{U \times 100}{U_o} \tag{1}$$

where U=total enzyme activity recovered in the support and U_o=enzyme units offered for immobilization.

Protein

Protein content was determined using the method according to Bradford [10].

Electrophoresis

The electrophoresis analysis of the free lipases (L036P and Piccantase R8000, both from *R. oryzae*) was performed according to the methodology established by Alfenas et al. [11].

Hydrolytic Activities

The hydrolytic activities of free and immobilized lipase derivatives were assayed by the hydrolysis of olive oil emulsion at a fixed proportion oil/water 1:1, pH 7.0 and 37 °C [12]. Free fatty acids were titrated using an automatic titration Metrohm Model 794 Basic Titrino (Metrohm Pensalab Instrumentação Analítica Ltda, São Paulo, SP, Brazil). The equivalent point was determined using the mode “dynamic titulation” (dynamic equivalence point titration) of the equipment. One unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of free fatty acid per minute under the assay conditions.

Esterification Reactions of Butanol with Butyric Acid

Reaction systems consisted of heptane (20 mL), *n*-butanol (100 mM), butyric acid (110 mM), and immobilized lipase derivatives (0.50 g, d.wt). The mixture was incubated at

37° C for 24 h with continuous shaking at 150 rpm. The formed product were determined by gas chromatography (Varian 3800, Varian Inc., Corporate Headquarters, Palo Alto, CA, USA) using a 6 ft 5% DEGS on Chromosorb WHP, 80/10 mesh column (Hewlett Packard, Palo Alto, CA, USA) at 65 °C and hexanol as internal standard. Esterification activity was expressed as μmol L^{-1} of butyl butyrate formed per minute per gram of dry support, and it was calculated with basis on the angular coefficient of the curve of butyl butyrate as a function of the time in its linear region.

Interesterification Reactions of Tripalmitin with Triolein

The interesterification reactions were performed in closed reactors (100 mL), under agitation (170 rpm), at 40 °C for 72 h. The reaction medium was composed of 50 mL of tripalmitin (60 mM) and triolein (40 mM) using hexane as solvent and 5 wt.% of immobilized lipase in relation to the total reaction medium. To monitor the reaction progress, 500 mg samples were withdrawn at different intervals and kept under −18 °C until the analysis procedure. Free fatty acid concentrations were determined by adding 200 μL of samples in 10 mL of alcohol/ether (1:2 *v*/*v*) and titrating the solution with 0.01 M potassium hydroxide using phenolphthalein as indicator. Triglycerides were analyzed by gas chromatography (Varian, Model GC 3800), using a capillary column CP Sil 5CB, operating at conditions previously described by Freitas et al. [5]. The interesterification yield (%) was calculated according to Eq. 2:

$$I(\%) = \frac{(C_{C_{50}} + C_{C_{52}})_t}{(C_{C_{48}} + C_{C_{54}})_0} 100 \quad (2)$$

where $C_{C_{50}}$ e $C_{C_{52}}$=concentration (mM) of triglycerides having 50 and 52 carbons in the residues of fatty acids, respectively; $C_{C_{48}}$ e $C_{C_{54}}$=concentration (mM) of tripalmitin and triolein, respectively. The indexes "t" and "0" represented the concentrations at given time and at the initial reaction, respectively.

Results and Discussion

Properties of the Evaluated Lipases and Immobilization Yield

All the seven evaluated food grade lipases were characterized in their free form in terms of hydrolytic activity, protein value (mg/g), and specific activity (μmol/mg protein). These results are shown in Table 1, which also presented the classification of these lipases in relation to their specificity and regiospecificity, according to the literature [13, 14].

Values for hydrolytic activity varied between 3,063 and 82,732 μmol/g min and protein content was in the range from 59.65 to 133.63 mg/g, indicating that these lipase preparations had distinct degree of purity (specific activity varied in the range from 25.39 to 1386.96 μmol/mg of protein). The highest purity was found for Lipomod™ 34P lipase, while Piccantase A lipase had the lowest purity. However, for biocatalytic applications, crude lipases are usually preferred due to their low cost and stability, since these commercial preparations contain sugar and other inert carriers.

After characterization, the enzymes were immobilized on POS-PVA matrix. The carrier chosen has a structure correspondent to a reticular arrangement, as previously described [15], and can be considered as an interpenetrating polymer network. Previous work showed

Table 1 Properties of the different food grade lipases evaluated in this work in their free form.

Lipase source	Trade Name	Fatty acid specificity[a]	Regiospecificity[a] (*sn*)	Protein (mg/g)	Hydrolytic activity (μmol/g min)	Specific activity (μmol/mg protein)
Aspergillus niger	Lipase A	S, M, L	1, 3 >> 2	96.10	19,677	204.76
Mucor javanicus	Lipase M	M, L >>S	1, 3 > 2	120.86	12,946	107.12
Candida rugosa	Lipomod™ 34P	S >> M,L	Unspecific	59.65	82,731	1386.96
Rhizopus oryzae	Lipase L036P	M, L > S	1, 3 >>> 2	93.19	58,918	632.24
Penicillium roqueforti	Lipomod™ 338P	S, M, >> L	1, 3	80.28	10,817	134.75
Rhizomucor miehei	Piccantase A	S > M, L	1 > 3 >> 2	120.62	3,062	25.39
Rhizopus oryzae	Piccantase R 8000	M, L > S	1, 3 >>> 2	133.63	10,791	80.76

L long-chain fatty acids, *S* short-chain fatty acids, *M* medium-chain fatty acids

[a] Data from literature [13, 14]

the potential for immobilizing lipases from porcine pancreatic for application in the production of sugar fatty acid esters and biodiesel [8] and from *C. rugosa* for esterification reactions [9]. In another work [16], chemical modification of POS-PVA with sodium metaperiodate was adequate to immobilize lipase, mainly for use in organic medium.

Table 2 summarizes the results for the covalent immobilization of the evaluated lipases on POS-PVA with regard to the hydrolytic activity and coupling yield. Among the tested lipases, the Picantase R8000 showed the higher coupling yield (53%), producing immobilized lipase samples with average activity of 1,472 μmol/g min. All the other lipases resulted in coupling yields lower than 38%, being the lowest (29.26%) attained by lipase L036P.

The different values obtained can be related to the lipase sources evaluated, which may give different interactions between enzyme and carrier. The immobilization procedure makes use of sodium metaperiodate as activating agent to promote the oxidation of free OH– groups in POS-PVA matrix, resulting in aldehyde groups that can bind directly with the protein moiety without any spacer arm. This may expose the lipase active site to possible damaging interactions with the POS-PVA surface. This may also confer lower

Table 2 Hydrolytic activity and coupling yield (%) for immobilized lipases on POS-PVA carrier.

Lipase source	Trade name	Hydrolytic activity (μmol/g min)	Coupling yield (η %)
Aspergillus niger	Lipase A	1,443	31.91
Mucor javanicus	Lipase M	1,896	32.04
Candida rugosa	Lipomod™ 34P	5,945	33.84
Rhizopus oryzae	Lipase L036P	3,752	29.26
Penicillium roqueforti	Lipomod™ 338P	1,072	32.98
Rhizomucor miehei	Piccantase A	235	37.39
Rhizopus oryzae	Piccantase R 8000	1,472	53.34

activity recovered, since the presence of a spacer arm is expected to reduce the modification on the enzyme structure induced by interactions between support and protein [16].

As can also be observed, that maximum (53.34%) and minimum (29.26%) values for coupling yields corresponded to Piccantase R8000 and L036P, respectively, both lipases from *R. oryzae*. One possible explanation for this inconsistency can be associated to the suppliers, which sometimes produce different preparations of the same lipase intended for different application. In addition, lipases from different suppliers may be different due to separate patents on different strains of the same species. In fact, different biochemical properties have been already reported for lipases obtained from this microorganism, previously known by other names as *Rhizopus arrhizus* and *Rhizopus javanicus* [17, 18].

Moreover, even lipase preparations produced by the same microbial strain can present different isoforms, which may have differences regarding their biochemical behaviors. These isoforms can be produced simultaneously by the microorganism during the process production or even can be aroused after purification [19]. As isoforms correspond to different protein molecules, they can interact in distinct manner with the surface of the immobilization carrier.

In case of the Piccantase R8000 and L036P lipases, such differences were attributed to the distinct lipase molar mass found in each commercial preparation as verified by gel electrophoresis analysis. As displayed Fig. 1, Piccantase R8000 and L036P have molecular mass of 54 and 40 kDa, respectively.

Application of the Lipases in Esterification Reactions

The potential of the selected lipases was determined in the esterification reaction of *n*-butanol with butyric acid using heptane as solvent. The product of this reaction, butyl butyrate, is in high demand as a component of pineapple flavor in the food, beverage, and pharmaceutical industries [4, 20].

The formed butyl butyrate profiles are shown in Fig. 2, while other parameters are summarized in Table 3. As can be observed, all lipases were able to catalyze the esterification reaction, although at different rates. The best performance was obtained using the Lipomod™34P, corresponding to an esterification activity 240.63 μM g^{-1} min^{-1} (Table 3).

In relation to the *R. oryzae* lipases, while the Piccantase R8000 produced the lowest amount of butyl butyrate (about 7 mM), the L036P was able to form tenfold of this concentration (about 70 mM). Such apparent inconsistency may be due to the differences

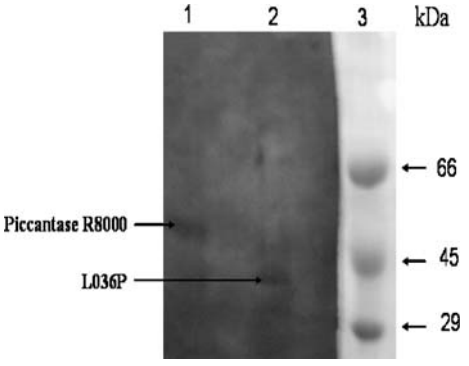

Fig. 1 Gel Electrophoresis of the lipases from *Rhizopus oryzae* from different suppliers. Characterization of the purified Piccantase R8000 (*column 1*), the purified L036P (*column 2*), and molecular mass markers (*column 3*)

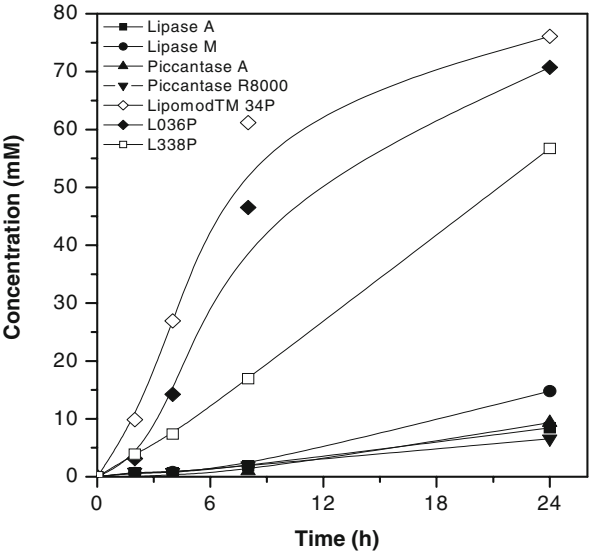

Fig. 2 Butyl butyrate production in the reactions catalyzed by different immobilized lipases on POS-PVA carrier

between these enzymes, as already explained. However, the behavior of the lipases from *R. oryzae* in this reaction cannot be fully understood. In contrast to the profound knowledge concerning factors governing selectivity of the lipase from *R. oryzae* in hydrolysis reactions, little information is available for the reverse reaction (esterification) in non-aqueous media [21]. Although lipases from *R. oryzae* have preference for medium and long chain fatty acids instead of short chain fatty acids (Table 1), L036P was able to catalyze the esterification of butyl butyrate and its esterification activity (171.14 μM/g min) was the second highest in comparison to the other evaluated enzymes.

Satisfactory results were also observed using lipase from *P. roqueforti* (L338P), with the production of 55 mM of butyl butyrate in 24 h reaction. This is in accordance with the preference of this enzyme for short and medium chain fatty acids, as stated in Table 1.

Regarding the other enzymes (Lipase A, Piccantase A, and Lipase M), the product concentrations were lower than 15 mM, correspondent to esterification activities at least 20 times lower than that the observed with Lipomod™ 34P, being the lowest (3.78 μM/g min) achieved by the Piccantase A.

Table 3 Esterification activity (μM/g min) in the butyl butyrate synthesis using the evaluated immobilized lipases on POS-PVA carrier.

Lipase source	Trade name	Esterification activity (μM/g min)
Candida rugosa	Lipomod™ 34P	240.63
Rhizomucor miehei	Piccantase A	3.78
Mucor javanicus	Lipase M	7.51
Rhizopus oryzae	Piccantase R 8000	8.63
Aspergillus niger	Lipase A	10.85
Penicillium roqueforti	Lipomod™ 338P	74.98
Rhizopus oryzae	Lipase L036P	171.14

Application of the Lipases in Interesterification Reactions

To evaluate the performance of the selected lipases immobilized on POS-PVA in interesterification reactions, the medium was composed of tripalmitin (PPP) and triolein (OOO) at concentrations of 60 and 40 mM, respectively. The curve profile for triglycerides concentrations as a function of time in the interesterification reactions catalyzed by immobilized lipases is shown in Fig. 3a–g. Product concentrations were evaluated by detection of all triglycerides present in the reaction medium in which the sum of carbon number in the fatty acid residues result in 50 (PPO, POP, and OPP) and 52 (POO, OPO, and OOP).

As can be observed, only the lipase L036P immobilized on POS-PVA (Fig. 3f) resulted in product concentration higher than 10 mM. For lipases M (Fig. 3b) and L338P (Fig. 3g), for example, no formation of triglyceride C_{50} was detected.

It is important to note that the consumption of the starting materials was independent of the product formation. This can be better understood by considering the role of water in the interesterification reactions. Although water is needed to maintain the enzymatic activity [6, 22], when in excess, it can favor the triglycerides hydrolysis instead of its re-esterification, with an accumulation of secondary products (monoglycerides, diglycerides and free fatty acids) in the

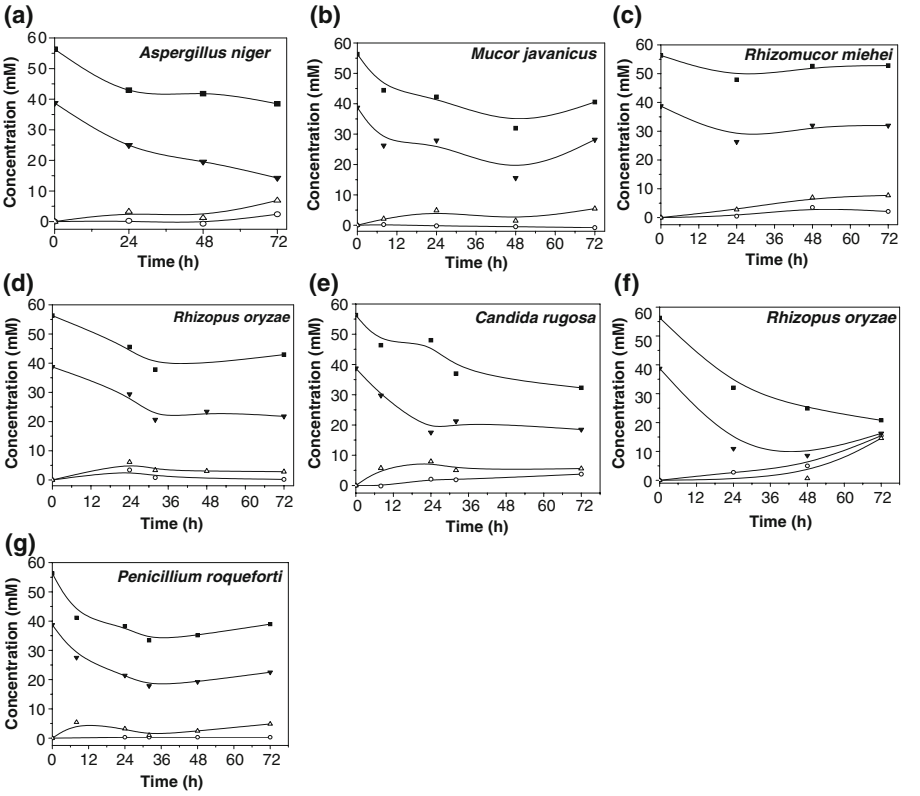

Fig. 3 Profile of the produced (*empty circle* C_{50}, *empty triangle* C_{52}) and consumed (*filled square* C_{48}, *filled inverted triangle* C_{54}) triglycerides concentration in the interesterification reactions of the tripalmitin with triolein, catalyzed by lipases A (**a**), M (**b**), Piccantase A (**c**), Piccantase R8000 (**d**), Lipomod™ 34P (**e**), L036P (**f**), and L338P (**g**), immobilized in POS-PVA. Triglycerides were identified according to the sum of the carbon number in the fatty acids residues

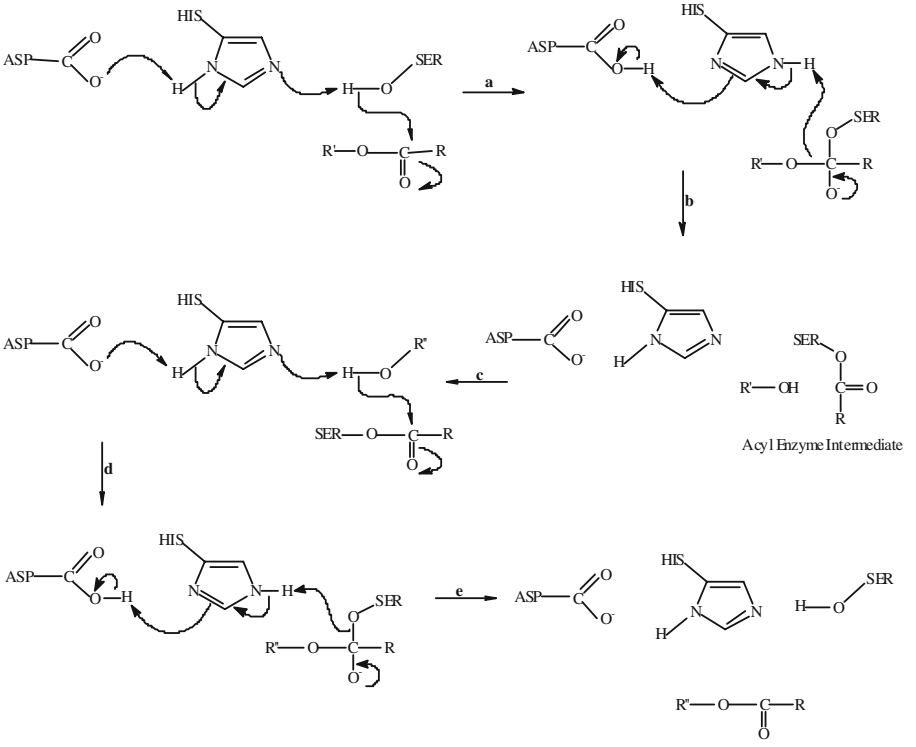

Fig. 4 Reactions involved in the catalytic site of the lipases: interesterification mechanism

reaction medium, as a consequence. Therefore, the amount of consumed substrate did not result in the accumulation of the triglycerides C_{50} and C_{52}.

Actually, the interesterification reaction can be considered as a special case of fatty acids transference that involves, in molecular level, sequential reactions of hydrolysis and

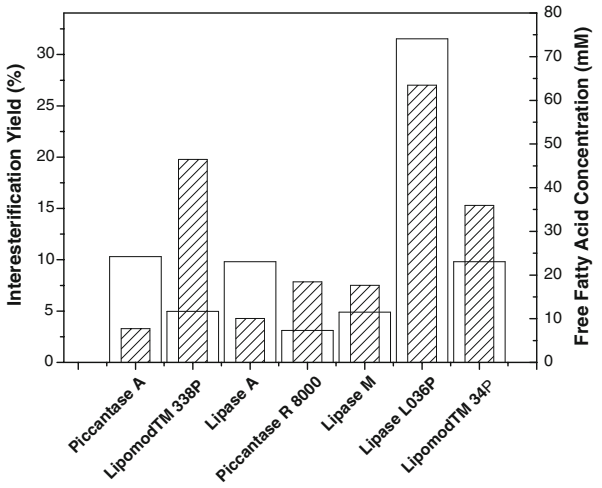

Fig. 5 Interesterification yield (%) (*blank bars*) and total free fatty acid concentration (mM) (*hachured bars*) in the interesterification reactions catalyzed by different food-grade commercial lipases

synthesis [23–25]. When the interesterification reaction is catalyzed by lipases, a mechanism according to Fig. 4 is followed. In this mechanism, acylation and desacylation occur in the lipase active site. The first stage of interesterification involves hydrolysis of triacylglycerols to produce diacylglycerols, monoacylglycerols, and free fatty acids. Accumulation of hydrolysis products will continue during interesterification until equilibrium is established [25]; however, when water is not in high quantity, re-esterification will occur and the interesterification will be favored.

The changes in the triglycerides profile allow calculating the interesterification yield as shown in Fig. 5, which also displayed the content of free fatty acid liberated at 72 h reaction.

The best interesterification performance was obtained by *R. oryzae* (L036P) lipase (Fig. 5) with typical values of around 31%. Such good performance may be related to its high hydrolytic activity (about 3,700 μmol/g min, Table 2) and preference for medium and long chain fatty acids. This lipase was also able to form the highest free fatty acid concentration, releasing about 63 mM in 72 h.

In relation to the other enzymes, much lower interesterification yields were achieved, with typical results varying from 3% to 10%. Among these enzymes, the lowest yield was obtained by Piccantase R8000. Lipase Lipomod™ 34P resulted in a high fatty acid value (about 36 mM), which can be explained by its high hydrolytic activity (Table 2). Furthermore, this lipase also showed the highest synthetic activity but its preference for short chain fatty acids may limit its performance in the interesterification reaction of tripalmitin with triolein.

Conclusions

Promising results were obtained showing the feasibility of using food grade lipases immobilized on POS-PVA previously activated with sodium metaperiodate as catalysts in different reactions of food industry's interest. All lipases were able to catalyze the synthesis of butyl butyrate in heptane, although at different rates. In this case, the highest product concentration value (76 mM) was achieved with the enzyme Lipomod™ 34P, which corresponded to an esterification activity of 240.63 μM/g min. In relation to the interesterification reactions, the best performance was obtained by *R. oryzae* (L036P) lipase. In the reaction catalyzed by this enzyme, the interesterification yield was in the order of 31%.

Acknowledgments The authors acknowledge the financial assistance from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors would like to give special thanks to Biocatalysts (Cardiff, England) and DSM Food Specialties (Delft, Netherlands) for kindly providing the lipase samples.

References

1. Hasan, F., Shah, A. A., & Hameed, A. (2006). *Enzyme and Microbial Technology*, *39*, 235–251.
2. Sharma, R., Chisti, Y., & Banerjee, U. C. (2001). *Biotechnology Advances*, *19*, 627–662.
3. Freitas, L., Bueno, T., Perez, V. H., Santos, J. C., & Castro, H. F. (2007). *World Journal of Microbiology & Biotechnology*, *23*, 1725–1731.
4. Santos, J. C., & De Castro, H. F. (2006). *World Journal of Microbiology & Biotechnology*, *22*, 1007–1011.

5. Freitas, L., Perez, V. H., Santos, J. C., & Castro, H. F. (2007). *Journal of the Brazilian Chemical Society*, *18*, 1360–1366.
6. Bornscheuer, U. T., Bessler, C., Srinivas, R., & Krishna, S. H. (2002). *Trends in Biotechnology*, *20*, 433–437.
7. Bruno, L. M., Coelho, J. S., Melo, E. H. M., & Lima-Filho, J. L. (2005). *World Journal of Microbiology & Biotechnology*, *21*, 189–192.
8. Paula, A. V., Urioste, D., Santos, J. C., & Castro, H. F. (2007). *Journal of Chemical Technology and Biotechnology*, *82*, 281–288.
9. Paula, A. V., Moreira, A. B. R., Braga, L. P., Bruno, L. M., & Castro, H. F. (2008). *Química Nova*, *31*, 35–40.
10. Bradford, M. M. (1976). *Analytical Biochemistry*, *72*, 248–254.
11. Alfenas, A. C, Peters-Robinson, I., & Brune, W. (1991). *Eletroforese de proteínas e isoenzimas de fungos e essências florestais, Viçosa, MG*.
12. Soares, C. M. F., Castro, H. F., Moraes, F. F., & Zanin, G. M. (1999). *Applied Biochemistry and Biotechnology*, *77/79*, 745–757.
13. Xu, X. (2000). *European Journal of Lipid in Science and Technology*, *102*, 287–303.
14. Saerens, K., Descamps, D., & Dewettinck, K. (2008). *Biotechnology Letters*, *30*, 311–315.
15. Santos, J. C., Paula, A. V., Rocha, C. G. F., Nunes, G. F. M., & Castro, H. F. (2008). *Journal of Non-crystalline Solids*, *354*, 4823–4826.
16. Santos, J. C., Mijone, P. D., Nunes, G. F. M., Perez, V. H., & Castro, H. F. (2008). *Colloids and Surfaces. B*, *61*, 229–236.
17. Benzonana, G. (1974). *Lipids*, *9*, 166–172.
18. Uyttenbroeck, W., Hendriks, D., Vriend, G., De Baere, I., Moens, L., & Scharpe, S. (1993). *Biological Chemistry Hoppe-Seyler*, *374*, 245–54.
19. Sayari, A., Frikha, F., Miled, N., Mtibaa, H., Ali, Y. B., Verger, R., et al. (2005). *Federation of European Biochemical Societies Letters*, *579*, 976–982.
20. Varma, M. N., & Madras, G. (2008). *Journal of Chemical Technology and Biotechnology*, *83*, 1135–1144.
21. Irmer, S., Potthoff, A. P., Kovac, A., Bucher, J., Stadler, P., Haalck, L., et al. (2000). *European Journal of Lipid in Science and Technology*, *102*, 173–180.
22. Hasegawa, S., Azuma, M., & Takahashi, K. (2008). *Enzyme and Microbial Technology*, *43*, 309–316.
23. Ghazali, H. M., Hamidah, S., & Che-Man, Y. B. (1995). *Journal of the American Oil Chemists' Society*, *72*, 633–639.
24. Graille, J. (1999). *Lipids*, *6*, 365–372.
25. Marangoni, A. J. (2002). In T. M. Kuo, & H. W. Gardner (Eds.),*Lipases: structure, function, & properties: Lipid biotechnology* p. 716 pp. United States of America: Marcel Dekker.