J Ind Microbiol Biotechnol (2012) 39:289–298
DOI 10.1007/s10295-011-1027-3

ORIGINAL PAPER

Immobilization of *Candida rugosa* lipase on poly (3-hydroxybutyrate-co-hydroxyvalerate): a new eco-friendly support

Rebeca Y. Cabrera-Padilla · Milena C. Lisboa · Alini T. Fricks · Elton Franceschi · Alvaro S. Lima · Daniel P. Silva · Cleide M. F. Soares

Received: 23 June 2011 / Accepted: 8 August 2011 / Published online: 26 August 2011

Abstract The overall objective of this study is to evaluate the morphological [scanning electron microscopy (SEM)], physicochemical [differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), chemical composition analysis, Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR)], and biochemical properties of *Candida rugosa* lipase (CRL) immobilized on a natural biopolymer poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV) in aqueous solution. CRL was immobilized by physical adsorption with efficiency of 30%. Compared with free CRL enzyme, there were slight changes in immobilized CRL activity as a function of temperature (from 37°C to 45°C), but a similar optimal pH value of 7.0. Inactivation rate constants for immobilized CRL enzyme were 0.009 and 0.334 h^{-1}, and half-lives were 77 and 2 h at 40°C and 60°C, respectively. Kinetic parameters obtained for immobilized CRL include the Michaelis–Menten constant of $K_m = 213.18$ mM and maximum reaction velocity of $V_{max} = 318.62$ U/g. The operational stability of immobilized CRL was tested repeatedly, and after 12 cycles of reuse, the enzyme retained 50% activity. Based on our results, we propose that PHBV-immobilized CRL could serve as a promising biocatalyst in several industrial applications.

Keywords *Candida rugosa* lipase · Immobilization · PHBV · Adsorption · Kinetic parameters

Electronic supplementary material The online version of this article (doi:10.1007/s10295-011-1027-3) contains supplementary material, which is available to authorized users.

R. Y. Cabrera-Padilla · M. C. Lisboa · A. T. Fricks · E. Franceschi · A. S. Lima · D. P. Silva · C. M. F. Soares
Universidade Tiradentes, Av. Murilo Dantas, 300, Bairro Farolândia, Aracaju, SE 49032-490, Brazil

A. T. Fricks · E. Franceschi · A. S. Lima · D. P. Silva · C. M. F. Soares (✉)
Instituto de Tecnologia e Pesquisa, Av. Murilo Dantas, 300, Prédio do ITP, Bairro Farolândia, Aracaju, SE 49032-490, Brazil
e-mail: cleide.soares@pq.cnpq.br

Introduction

Enzymes offer a distinct advantage over chemical catalysts, because of their specificity and stereoselectivity, catalytic efficiency under mild conditions, biodegradability, and limited formation of side-products [2]. In addition, enzymes can catalyze reactions in different states: as individual molecules in solution, in aggregates with other entities, and while attached to surfaces. The attached, or immobilized, state has been of particular interest to efforts aimed at exploiting enzymes for technical purposes [10]. In fact, immobilization of enzymes already plays an important role within applied biotechnology. The main reason for immobilizing enzymes is that it enables isolation of the reaction products and reuse of the biocatalyst, thus increasing productivity [19].

The characteristics of the matrix or support are important factors which influence the performance of the immobilized enzyme system. Supports can be classified as inorganic or organic, according to their chemical composition. Organic supports can be made from natural or synthetic polymers [10]; for example, lipase has been successfully immobilized onto many different types of carriers, such as Celite, sporollenin, and silica [27, 31]. Free or immobilized lipases are versatile biocatalysts for hydrolysis, transesterification, esterification, and other reactions, which are useful in industrial applications [23]. The most common procedures for enzyme immobilization

involve enzyme adsorption to a support matrix, because of its ease of use and low cost.

Contact forces between the support and the enzyme include hydrogen bonding, van der Waals forces, and hydrophobic interactions [31]. Each technology has been further optimized, and new materials have been developed for adsorption or encapsulation techniques [19, 21]. Several eco-friendly supports have been reported in the literature for enzyme immobilization, such as chitin, chitosan, acetyl cellulose, dextran, alginate, and others [8, 10, 17, 30].

Although there is still no universal support that is suitable for all enzymes and all applications, any material that is to be considered as an enzyme support must fulfill certain requirements: high affinity for proteins, availability of reactive functional groups (for direct reaction with enzymes or chemical modification of the support), mechanical stability, rigidity, feasibility of regeneration, and high loading capacity. Depending on the application, nontoxicity and biodegradability may also be required [8].

Various eco-friendly supports have not yet been tested for enzyme immobilization, for example, poly(hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV). These polyesters are produced biologically by various microorganisms in nature, as reserve materials stored as intracellular granules. In addition, they have been produced on an industrial scale, using bacteria such as *Alcaligenes eutrophus*. These commercially available biopolymers have attracted much attention for use in agriculture, marine, and medical applications. The major advantages of these thermoplastic polymers are their biocompatibility and biodegradability [29].

However, PHB is stiff and brittle, restricting its range of application. On the other hand, PHB copolymers with 3-hydroxyvalerate (PHBV) are less stiff, tougher, and crystalline [5]. Recently, use of poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV) in biomedical applications has increased, mainly because of the fact that it is possible to prepare an appropriate controlled drug delivery system that gradually degrades in the body [3]. Based on the above properties, we propose that PHBV could serve as a good alternative enzyme immobilization support because of its biocompatibility, biodegradability, strength, easy reabsorption, non-toxicity, and eco-friendliness.

To the best of our knowledge, no reports exist in the literature on use of PHBV as a lipase immobilization support. In this study, the biochemical, morphological, and physicochemical properties of PHBV-immobilized lipase have been studied. The effect of temperature and pH were investigated, and thermal stability, reusability, and kinetic properties were analyzed. In addition, PHBV-immobilized lipase was characterized by scanning electron microscopy (SEM), thermogravimetry (TGA), differential scanning calorimetry (DSC), infrared spectroscopy (FTIR), and ^{13}C nuclear magnetic resonance.

Materials and methods

Enzyme and chemicals

Lipase from *Candida rugosa* (CRL, type VII) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nominal lipase activity was 2,381 U/g. Poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV), a natural biopolymer, was used as a support for CRL immobilization. PHBV was kindly supplied by PHB Industrial S.A. Hexane and acetone were obtained from Isofar (Rio de Janeiro, Brazil); ethanol 95% was obtained from Vetec (Rio de Janeiro, Brazil); gum Arabic was obtained from Cromoline (São Paulo, Brazil); olive oil was purchased at a local market. Other chemicals were of analytical grade and used as received.

Activity of lipase in hydrolysis of emulsified olive oil

The hydrolytic activities of free and immobilized lipase were assayed by the olive oil–water emulsion method, according to the modification proposed by Soares et al. [25]. The substrate was prepared by mixing 50 mL olive oil with 50 mL gum Arabic solution (7% w/v). A reaction mixture containing 5 mL of the emulsion, 2 mL 0.1 M sodium phosphate buffer (pH 7.0), and either free (100 mg) or immobilized (100–250 mg) enzyme was incubated for 5 min for free and 10 min for immobilized at 37°C under stirring. The reaction was stopped by addition of 2 mL acetone–ethanol–water solution (1:1:1). Liberated fatty acids were titrated with 0.01 M potassium hydroxide solution in the presence of phenolphthalein as an indicator. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol free fatty acid per min (μmol min^{-1}) under assay conditions (37°C, pH 7.0, 150 rpm). Analyses of hydrolytic activities performed on free and immobilized lipase were used to determine the coupling yield η (%) according to Eq. 1.

$$\eta(\%) = \frac{U_S}{U_0} \times 100 \quad (1)$$

in which U_S corresponds to the total enzyme activity recovered on the support and U_0 represents the enzyme units offered for immobilization.

Effect of solvents on lipase activity

To select the optimal solvent for enzyme immobilization on the PHBV support, different samples of 0.1 g free CRL enzyme were incubated in the presence of dichloromethane,

chloroform, hexane, and heptane for 10, 20, 40, and 60 min at 30°C and 40°C. After the end of the incubation period, the supernatant was removed and the remaining solvent was evaporated. Then the activity was determined, as described previously [25].

Enzyme immobilization

Control tests to verify the stability of support (PHBV) in the presence of lipase were carried out under similar conditions to test the hydrolytic activity of the enzyme over the support, in the absence of olive oil at different incubation times (5, 10, 15, 30, 60 min, 24, and 48 h). Tests were conducted with 0.1 g PHBV, 0.1 g free CRL, 2 mL 0.1 M sodium phosphate buffer (pH 7.0), and 5 mL gum Arabic solution (7% w/v) (test 1). Another test was performed with 0.1 g PHBV, 0.1 g free CRL, and 7 mL sodium phosphate buffer (test 2). All tests were performed at 37°C with agitation. The analysis of control tests was carried out by titration with 0.01 M potassium hydroxide solution to identify the presence of acids produced by support degradation.

Lipase from *Candida rugosa* was immobilized by physical adsorption on PHBV using a procedure modified from Soares et al. [28]. Briefly, 20 mL hexane was added to 2 g support with vigorous agitation at room temperature for 2 h, then 20 mL enzymatic solution (mass of the enzyme solubilized in 20 mL 0.1 M sodium phosphate buffer pH 7.0) was added to the hexane and support suspension and agitated for 2 more hours. The enzyme–support support was then incubated for 24 h at 4°C. Immobilized lipase was recovered by vacuum filtration coupled with repeated washes with hexane. The water content (dry weight) and enzyme activity of the immobilized biocatalyst were then quantified. Filtrates and washes were collected and used for activity determination. The prepared immobilized lipase was then stored at 4°C until use.

To determine the optimal amount of lipase, we tested the effect of various amounts of enzyme/support ratios (0.15, 0.225, 0.3, 0.375, and 0.45) on immobilization.

Biochemical properties of free and immobilized lipase

Effect of pH and temperature on activity

The effect of pH on the relative activity of free and immobilized lipase was assayed in phosphate buffer (0.1 M) at pH values ranging from pH 5.0 to 9.5.

The effect of temperature on the relative activity of the free and immobilized lipase was determined at pH 7.0 in the temperature range from 30°C to 70°C. Relative activities for both assays were calculated as the ratio of enzyme activity measured at different temperatures to the maximal activity of the enzyme measured as described previously [25].

Thermal stability

Free and immobilized lipase preparations were stored in sodium phosphate buffer solutions (0.1 M, pH 7.0) for 4 h at 40°C and for 2 h at 60°C, respectively. Samples were periodically withdrawn for activity assays. Residual activities were calculated as the ratio of the activity of enzyme measured after incubation to the maximal activity of the enzyme.

The thermal inactivation constant (k_d) and half-life ($t_{1/2}$) for immobilized CRL were calculated using Eqs. 2 and 3, respectively, according to the literature [7, 26]

$$A_{in} = A_{in_0} \exp(-k_d \cdot t) \quad (2)$$

$$t_{1/2} = \ln(0,5)/-k_d \quad (3)$$

where A_{in} is the residual activity after heat treatment for a period of incubation (U), A_{in_0} is the initial enzyme activity (U), k_d is the inactivation constant (h^{-1}), and $t_{1/2}$ is the half-life (h).

Reusability of immobilized lipase

The operational stability and reusability of the immobilized system was determined by conducting hydrolysis reactions in consecutive batches using the same immobilized enzyme. Each batches consisted of 10 min of hydrolysis reaction at temperature of 37°C and pH 7.0. After each batch, the immobilized enzyme was washed with hexane once and reused for the next cycle of hydrolysis. This procedure was repeated for 20 cycles.

Determination of K_m, V_{max}, and catalytic efficiency

To calculate the Michaelis–Menten constant (K_m and V_{max}), reaction systems were prepared containing fatty acids at concentrations ranging from 37 to 2,232 mM, obtained from emulsions containing different proportions of olive oil (1–60%) and aqueous solution of gum Arabic (7% w/v). Initial hydrolysis reaction rates, catalyzed by free and immobilized CRL, were determined according to methodology described in "Activity of lipase in the hydrolysis of emulsified olive oil" The apparent values of K_m and V_{max} were calculated by nonlinear fitting using the program Origin® 8.0. Catalytic efficiency were calculated using the program Graphpad Prism 5.0

Morphological and physicochemical properties

Scanning electron microscopy (SEM) was used to characterize the free enzyme (CRL), support (PHBV), and immobilized biocatalyst (PHBV-CRL) samples.

Sample weight loss upon heating was determined using a thermogravimetric analysis (TGA) apparatus (Shimadzu

TGA-60 thermogravimetric analyzer) over the temperature range of 30–550°C. Samples with mass variation of 2–6 mg were put in a sealable aluminum pan, and analyzed under nitrogen atmosphere with a heating rate of 20°C/min.

Differential scanning calorimetry data were recorded using a Shimadzu DSC-60H differential scanning calorimeter. Support (PHBV) and immobilized biocatalyst (PHBV-CRL) samples with mass variation 2–6 mg were put in a sealable aluminum pan, which was heated from 30°C to 200°C at a rate of 20°C/min.

Free enzyme (CRL), support (PHBV), and immobilized biocatalyst (PHBV-CRL) samples were also analyzed by Fourier-transform infrared (FTIR) (BOMEM MB-100 spectrophotometer). FTIR spectra were obtained over the wavelength range from 400 to 4,000 cm^{-1}.

For the support (PHBV) and immobilized biocatalyst (PHBV-CRL) samples, the structure was characterized by recording ^{13}C NMR spectra. Solid-state ^{13}C cross-polarization magic-angle spinning (CP-MAS) NMR spectra were obtained on an NMR Inova 400 spectrometer at 75 MHz. The samples were spun at a magic angle of ca. 100 kHz. The pulse interval time was 3 s, and the pulse duration was 0.050 s. For this atom type, chemical shifts was measured using Si $(CH_3)_4$ as reference and hexamethylbenzene (17.13 ppm) for C.

Results and discussion

Effect of solvents on lipase activity

The choice of the solvents heptane and hexane to study the effect on lipase activity in organic solvents was motivated by the routine use of these solvents in adsorption immobilization techniques. The effect of solvent on lipase activity was also studied using dichloromethane and chloroform because PHBV is soluble in these solvents and another technique of enzyme encapsulation in PHBV (core–shell formation) is under study by our group.

Figure 1a and b shows stability results for free CRL in the presence of the four different organic solvents at 30°C and 40°C, respectively. Based on these results, the enzyme showed similar activities in the presence of hexane and heptane at the two temperatures studied. Hexane was selected to perform immobilization of CRL on the PHBV support by physical adsorption, because hexane is generally used as a solvent for the immobilization technique by adsorption as reported in the literature [6, 18, 34].

Immobilization of lipase by physical adsorption on PHBV

To evaluate the loading capacity of the PHBV support, a particle size distribution between $100 > X \geq 200$ mesh was evaluated. To determine the optimal amount of lipase, we studied the immobilization of various amounts of enzyme on 2.0 g PHBV support. The effect of enzyme/support ratio on activity is shown in Fig. 2. Lipase activity was significantly increased when the enzyme/support ratio was increased from 0.15 to 0.3 (w/w), while above a ratio of 0.3 (w/w) the activity remained practically constant, due to saturation of the PHBV support with lipase. Because the highest activity (325.75 U/g) was achieved when a ratio of 0.3 was used, this enzyme/support (w/w) ratio was selected for immobilization of CRL on PHBV. To obtain this ratio, 926 $U_{enzyme}/g_{support}$ were applied. For these conditions, the immobilization yield obtained was 30%, considered satisfactory result when compared with other enzymes immobilized on several types of support such as commercially available acrylic polymers (recoveries from 8% to 20%) [4].

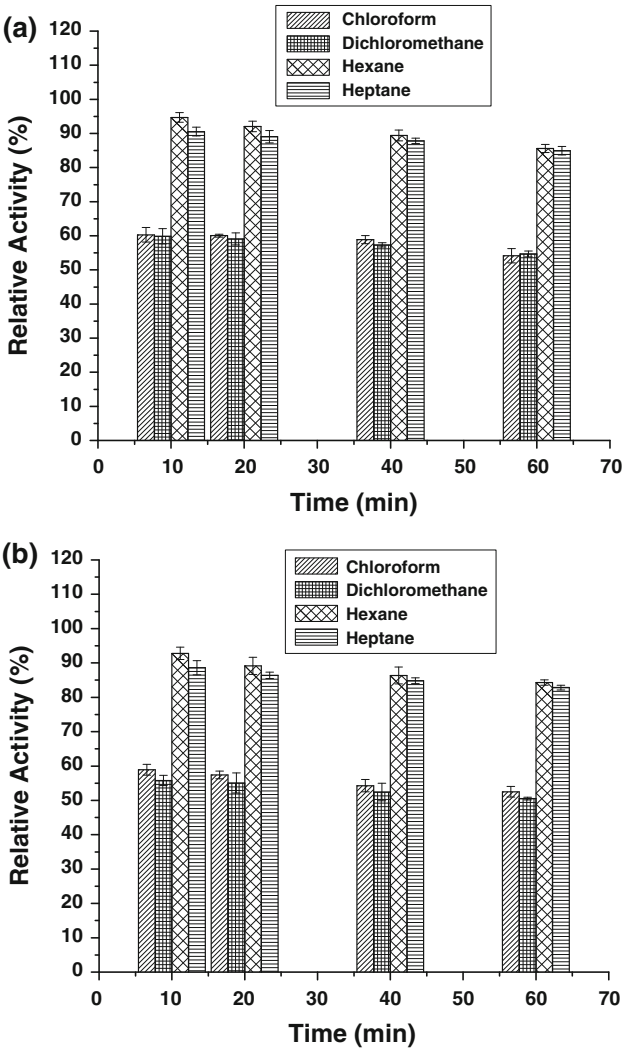

Fig. 1 Effect of solvents on the activity of free CRL at **a** 30°C and **b** 40°C. *Error bars* are standard deviations from triplicates

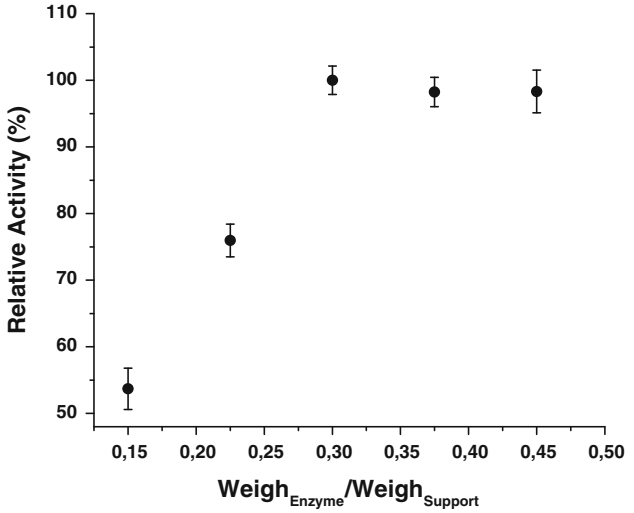

Fig. 2 Relative activity as a function of enzyme/support ratio during immobilization of CRL on PHBV. *Error bars* are standard deviations from triplicates

Biochemical properties

Effect of pH and temperature on lipase activity

It is well known that enzyme immobilization on insoluble supports has a variety of effects on the ionization state and enzyme dissociation. In particular, pH can have a profound effect on hydrolytic activity after the immobilization process. In fact, pH is one of the most influential parameters for enzymatic activity in an aqueous medium. In addition, immobilization is likely to result in a conformational change in the enzyme, which may lead to enzyme inactivation, and may cause a shift in the pH dependence of the enzyme activity. This pH shift depends mainly on the method of immobilization and the interaction between the enzyme and support.

The effect of pH on the relative activity of free and immobilized CRL during hydrolysis of emulsified olive oil was determined between pH 5.0 and 9.0, and the results are shown in Fig. 3a. The maximum value of relative activity was observed at pH 7.0 for immobilized CRL, the same pH value obtained for the free enzyme. The pH values obtained are similar to results described in the literature for silica-matrix-encapsulated CRL [28].

In general, the immobilized enzyme was less stable to pH effects than the free enzyme, despite having the same optimal pH (7.0); below or above this pH value, the immobilized enzyme activity exhibited an abrupt drop that was not observed around the maximum region of the free enzyme, suggesting that the immobilization technique caused negative changes to the conformation of the enzyme. Similar results have been previously reported for *Candida rugosa* lipase immobilized on electrospun

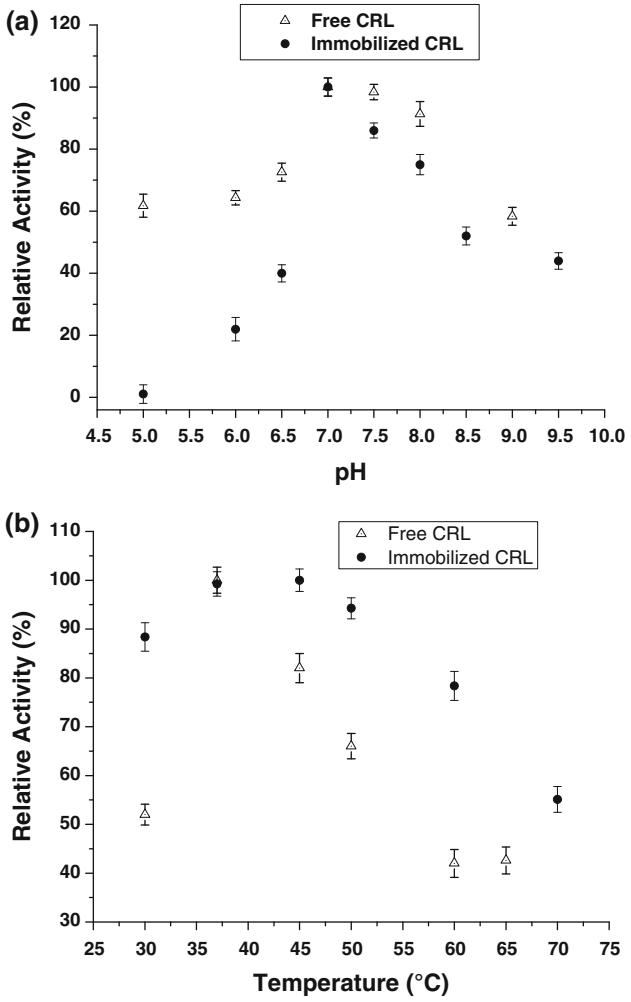

Fig. 3 Effect of **a** pH and **b** temperature on the relative activity of free and immobilized CRL. *Error bars* are standard deviations from triplicates

cellulose nanofiber membrane [11] and cyclomaltodextrin glucanotransferase from *Thermoanaerobacter* immobilized by sol–gel technique [1].

The resistance of immobilized lipase to temperature is an important potential advantage for practical applications of this enzyme. The temperature dependence of the relative activity of free and immobilized lipase was investigated and compared by measuring hydrolysis of emulsified olive oil at different temperatures, and the results are shown in Fig. 3b.

As can be seen in Fig. 3b, the optimal temperature for the free enzyme was approximately 37°C, while for the immobilized enzyme the optimal temperature was in the range of 37–45°C. In other words, immobilized CRL has increased tolerance to heat compared with free CRL. Similar results were reported by Tutar et al. [31] for CRL immobilized on sporopollenin and Li et al. [13] for PPL immobilized on rod-like SBA-15.

One of the main reasons for enzyme immobilization is the anticipated increase in enzyme stability. Immobilized enzymes display resistance to various deactivating forces, due to their restricted conformational mobility [20]. This was may be due to conformational limitations to enzyme movement as a result of electrostatic interactions and hydrogen bonding between the enzyme and the support. Thus, immobilized enzymes show increased catalytic activities at higher reaction temperatures [33]. At higher temperatures, free lipase could easily undergo thermal denaturation, while immobilized lipase is protected, probably because of the more rigid conformation provided by the support, and therefore is able to retain its catalytic activity.

Thermal stability

The thermal stability of free and immobilized CRL was investigated and compared by measuring the hydrolysis of emulsified olive oil by lipase at 40°C and 60°C for different reaction times. As shown in Fig. 4, the thermal stability of the immobilized enzyme was greater than that of the free enzyme. The thermal stability of lipase has been shown to correlate with its structure [35]. At 40°C, the residual activity of the free enzyme was approximately 21%, while the residual activity of the immobilized CRL was 94% after 4 h temperature exposure. At 60°C, the free enzyme lost all of its activity after 3 h exposure, while PHBV-CRL still retained residual activity of 18% after 4 h exposure at this temperature.

It is possible that the enhanced hydrophobic interactions offered by the PHBV support could increase the thermal stability of immobilized CRL. However, aggregation of enzyme on the support could also weaken the hydrophobic characteristic of the support. In any case, based on the above results, the thermal stability of the CRL enzyme was clearly improved upon immobilization, in agreement with reported results [14, 35].

Data from the natural logarithm of hydrolytic activity versus time were adjusted to calculate the inactivation rate constant (k_d) and half-life ($t_{1/2}$), using Eqs. 2 and 3. The values calculated at temperature of 40°C were $k_d = 0.009\ h^{-1}$, $t_{1/2} = 77$ h and at 60°C were $k_d = 0.334\ h^{-1}$, $t_{1/2} = 2.1$ h.

Reusability of immobilized lipase

The reusability of immobilized enzymes is very important for various applications, especially in industrial applications. Figure 5 shows the variation in relative activity of immobilized CRL after multiple cycles of reuse. We found that immobilized CRL can be reused up to 12 times, while retaining 50% of its initial activity. Although somewhat decreased immobilized CRL activity was found with each successive reaction, this may be due to CRL leaching out from the surface of PHBV during the multiple soaking, separation, and washing with hexane steps employed during the recycling reaction, because the enzyme is only attached by weak interaction forces [32]. Li et al. [13] reported that activity of PPL immobilized on rod-like SBA-15 mesoporous material by adsorption decreased with reuse, retaining 32% of its original activity after five reuses. Huang et al. [11] also reported decreased activity with covalent immobilization of *Candida rugosa* lipase on a cellulose nanofiber membrane, the remaining activity being about 30% after eight cycles of batch operation. Based on our results, lipase immobilized on PHBV is very suitable for commercial applications because of its easy recovery from the reaction system and efficient reuse.

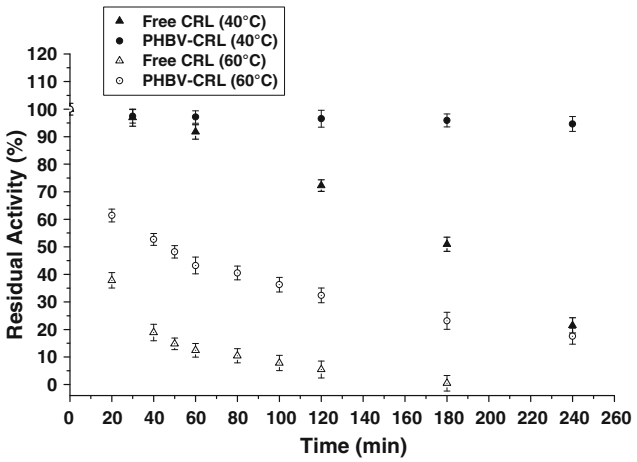

Fig. 4 Thermal stability of free and immobilized CRL (incubated at 40°C and 60°C). *Error bars* are standard deviations from triplicates

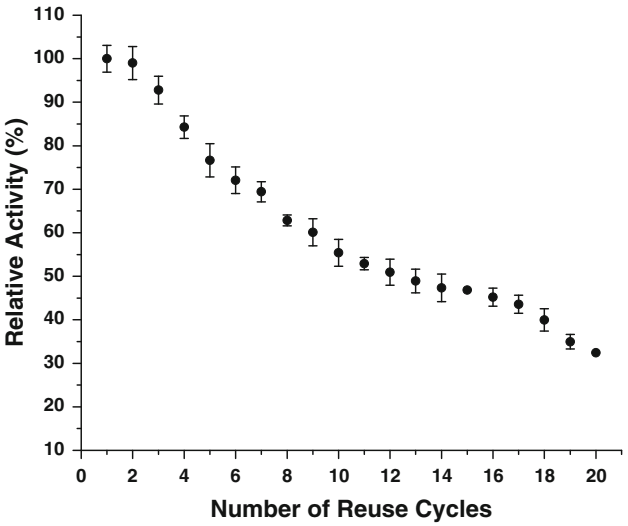

Fig. 5 Relative activity of immobilized CRL as a function of reuse. *Error bars* are standard deviations from triplicates

Kinetic parameters

The kinetics of the hydrolytic activity of the free enzyme (CRL) and immobilized biocatalyst (PHBV-CRL) were investigated at various concentrations of olive oil substrate (at 37°C, pH 7.0). The Michaelis–Menten equation was used to fit the kinetic parameters and data from the initial reaction rate to evaluate the constants, K_m and V_{max}, using the program Origin® 8. The K_m and V_{max} of the free enzyme were 835.59 mM and 4,354.61 U/g, respectively, whereas the apparent K_m and apparent V_{max} of the immobilized enzyme were 213.18 mM and 318.62 U/g, respectively. Lipase immobilized on PHBV showed a decrease in apparent K_m. The K_m value is known as the criterion for the affinity between enzyme and substrate. The higher affinity for substrate obtained in the case of immobilized enzyme could be explained by the microenvironment of the immobilized enzyme being hydrophobic, with low water content, whereby the substrate which is also hydrophobic is attracted, thus justifying the obtained values for K_m. However, in the case of free enzyme, the greater extent of hydrophilicity of the medium reduces the attraction between the enzyme and substrate. This behavior was confirmed by the determination of the catalytic efficiency, where the values obtained were 0.45 and 0.14 for immobilized and free enzyme, respectively. This is, however, not reflected in the apparent V_{max}. Lipase immobilized on PHBV showed a decrease in apparent V_{max}. These results suggest that the enzyme could have undergone a conformational change during immobilization on PHBV, affecting the rate of the reaction. A change in orientation could lead to improper positioning of the active sites for binding to the substrate. Besides the not so favorable conditions for increased reaction rate by the immobilized enzyme in this case could be because the substrate being insoluble, the immobilized enzyme may act only on the available fraction while the free enzyme could interact freely with the substrate [12]. Similar results of a decreased apparent K_m and decreased apparent V_{max}, which is not usually seen, was obtained for immobilized *Candida rugosa* lipase by Montero et al. [16], *Thermomyces lanuginosus* lipase immobilized through crosslinking using glutaraldehyde and hen egg white by Karimpil et al. [12], and for enzymes co-immobilized onto alkylamine glass beads by Minakshi and Pundir [15].

Morphological and physicochemical properties

Scanning electron microscopy (SEM)

SEM was used to characterize the morphology of the free enzyme (CRL), support (PHBV), and immobilized biocatalyst (PHBV-CRL). The resulting SEM photomicrographs are shown in Fig. 6. The image corresponding to PHBV-CRL shows a higher concentration of granules versus with PHBV alone, indicating the presence of the CRL enzyme immobilized on the support surface.

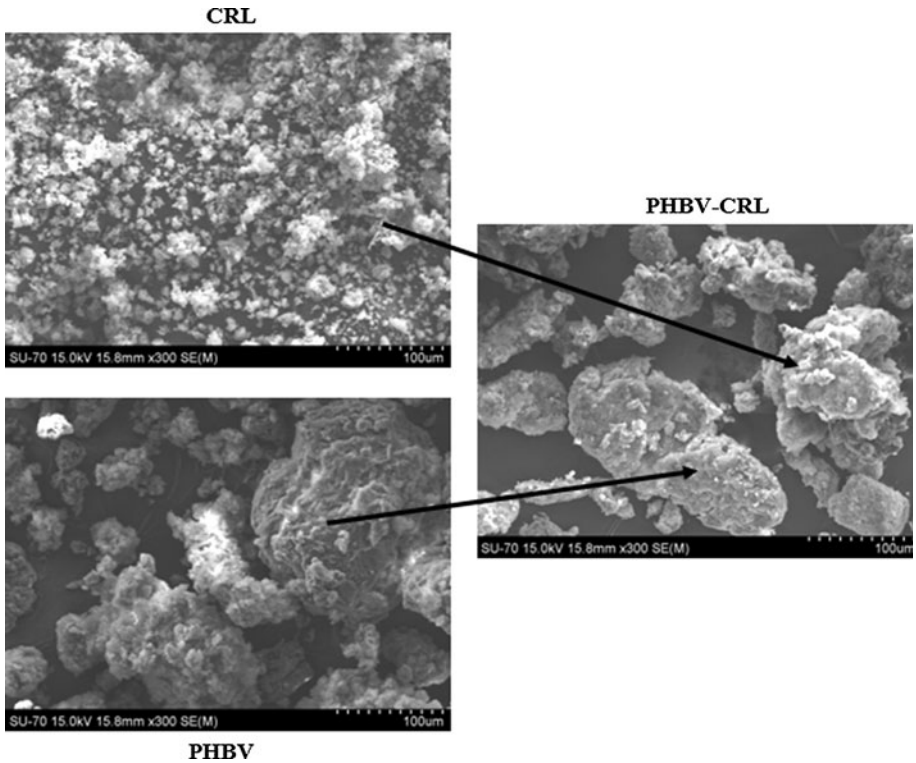

Fig. 6 Scanning electron microscopy of the free enzyme (CRL), support (PHBV), and immobilized biocatalyst (PHBV-CRL)

Differential scanning calorimetry (DSC)

DSC curves for the support (PHBV) and the immobilized biocatalyst (PHBV-CRL) showed similar behaviors, with both presenting a single peak for the temperature range studied, as shown in the supplementary material (Online Resource 1). For the PHBV, a melting temperature of $T_m = 166.6°C$ and enthalpy of fusion of $\Delta H_m = 84.59$ J/g were determined, being similar to those reported by Gonçalves et al. [9]. For the immobilized biocatalyst (PHBV-CRL), $T_m = 166.8°C$ and $\Delta H_m = 66.43$ J/g were found. Based on these data, immobilized PHBV-CRL absorbed less heat to reach the melting temperature compared with the PHBV support alone. This fact is possibly caused by differences in thermal conductivity between the immobilized biocatalyst and support, promoting a reduction in heat flow.

Thermogravimetric analysis (TGA)

TGA is an important tool that enables determination of the temperature range at which a heated sample undergoes a major conformational change, by means of monitoring its thermal weight loss profile. The weight loss curves were divided into three regions: region I (0–200°C), region II (200–400°C), and region III (400–550°C).

Figure 7 shows that, in region I, free CRL loses mass from the beginning, while for the support (PHBV) and the immobilized biocatalyst (PHBV-CRL), the mass loss remained nearly constant. Region II shows high mass loss with increasing temperature for all three samples. Free CRL in this region experienced lower mass loss compared with the support and the PHBV-CRL, which displayed similar behaviors. For PHBV, the maximum degradation occurred at 340°C, with onset ($T_{initial}$) at 254°C, consistent

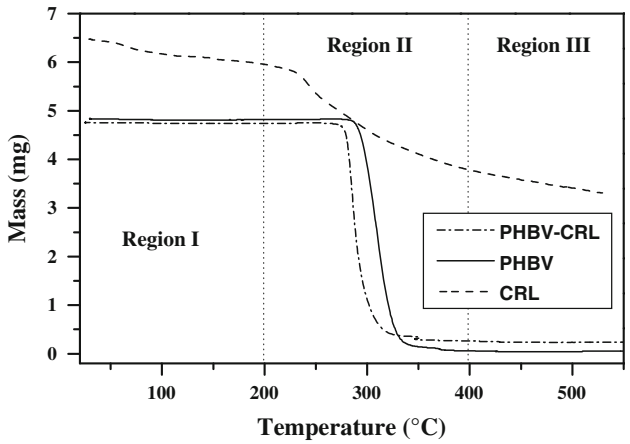

Fig. 7 Thermogravimetric curves for free enzyme (CRL), support (PHBV), and immobilized biocatalyst (PHBV-CRL)

with previous reports in the literature stating that PHBV is thermally unstable above 250°C. During PHBV thermal degradation, chain scission and hydrolysis was shown to lead to a reduction in molecular weight and the formation of crotonic acid [22]. For PHBV-CRL, the maximum degradation occurred at 315°C, with onset at 250°C. The immobilized biocatalyst (PHBV-CRL) is more stable than free CRL, as shown in region II of Fig. 7, and for free CRL, sharp weight loss was observed from the initial heating stage (regions I, II, and III).

In region III a slight variation in mass was observed after thermal degradation of both the support and the immobilized biocatalyst (PHBV-CRL). For the free CRL the mass loss was still substantial in region III. This fact is probably associated with the decomposition of organic compounds from the biocatalyst [28].

FTIR analysis

The FTIR spectra obtained for the free enzyme (CRL), support (PHBV), and immobilized biocatalyst (PHBV-CRL) are shown in Fig. 8.

The free enzyme displayed a typical FTIR protein spectrum, with bands in the range 1,650–1,540 cm^{-1} associated with characteristic primary and secondary amino groups (CONH). Although those bands are very faint in FTIR spectra of the immobilized biocatalyst, they reveal the presence of the CRL amino groups.

The FTIR spectra for PHBV and PHBV-CRL were similar, with a small increase in the number of bands for the immobilized biocatalyst versus the support, consistent with the presence of CRL enzyme on the support surface, verifying once again that the immobilization technique by physical adsorption was efficient.

In the FTIR spectrum of PHBV, bands assigned to C–O–C group stretching vibrations between 1,245 and 1,319 cm^{-1} are present, as well as bands associated with double bond C=O stretching, at 1,700–1,760 cm^{-1}, in agreement with results reported by Gonçalves et al. [9]. Bands were also present at approximately 1,240, 1,220, and 1,160 cm^{-1} (marked with arrows), which are sensitive to the degree of crystallization. In addition, bands at approximately 1,130, 1,090, and 1,020 cm^{-1} are also sensitive to the degree of crystallization, but to a lesser extent [22].

NMR analysis

^{13}C NMR spectra of the support (PHBV) and the immobilized biocatalyst (PHBV-CRL) also showed similar behavior, with a small displacement and decrease in peaks seen for the immobilized biocatalyst compared with the support, as shown in the supplementary material (Online Resource 2). The chemical shifts associated with the

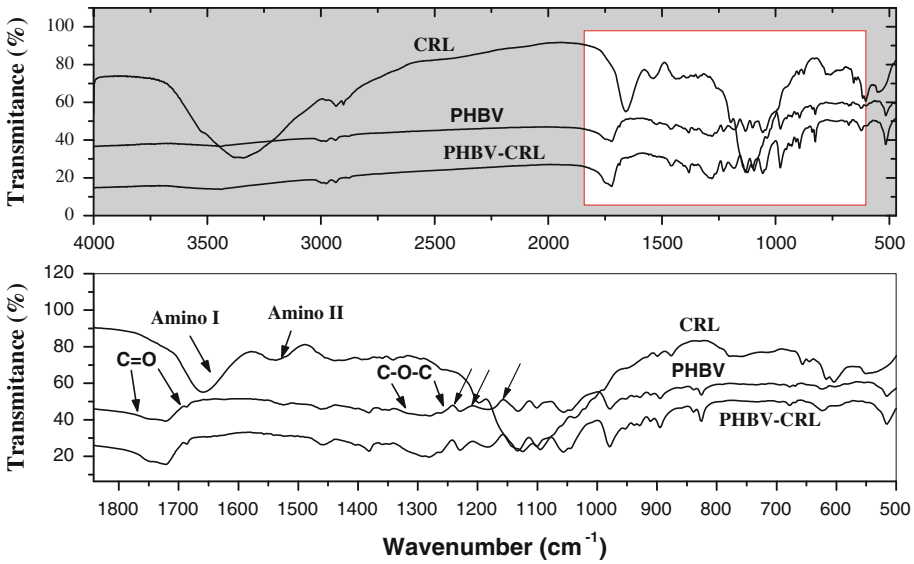

Fig. 8 FTIR spectra of the free enzyme (CRL), support (PHBV), and immobilized biocatalyst (PHBV-CRL)

resonance peaks observed in the spectrum of the PHBV support are similar to that reported by Slater et al. [24], however a small peak at approximately 117 ppm was identified, which is probably due to the presence of impurities in the composition of the support.

In the NMR spectra of the support (PHBV), four peaks of greater intensity were observed: peak 1, 2, 3, and 4. Peak 1 corresponds to a carbonyl group (C=O), peak 2 to a methylene group ($–CH_2–$), peak 3 to a methine group ($\equiv CH$), and peak 4 to a methyl group ($–CH_3$). In the NMR spectrum of the chemical structure of PHBV, we also observed various carbon resonances for the two monomers in the polymer, including side-chain methyl and methylene groups and the backbone chain methine and methylene groups. In addition, the spectrum for the carbonyl carbons is shown with assignments to ^{13}C-3-hydroxybutyrate-3-hydroxyvalerate (3HV, peak 5) and ^{13}C-3-hydroxyvalerate-3-hydroxybutyrate (3HB, peak 1) at approximately 169 ppm. This indicates a random sequence distribution of monomers as previously described in the literature [24].

Evaluation of biodegradability

The evaluation of possible degradation of the biopolymer was conducted during the immobilization process by physicochemical analysis and control tests in the same reaction conditions of hydrolysis with the immobilized enzyme.

The structure of the immobilized lipase and support was maintained as shown in the analysis according to the electronic supplementary materials (DSC in Online Resource 1 and NMR in Online Resource 2) and in this article (TGA in Fig. 7 and FTIR in Fig. 8).

Control tests in the same reaction conditions of hydrolysis of the immobilized enzyme at different time intervals showed the absence of acids produced by degradation of support. In this sense, we can affirm that the enzyme does not cause degradation of the biopolymer structure during the immobilization process and the hydrolysis reaction.

Conclusions

Novel eco-friendly supports, such as poly(hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV), have a promising future for enzyme catalysis in biotechnology, because they allow easy lipase immobilization through a simple, inexpensive process and are biodegradable. One of the most important aims of enzyme technology is to enhance the conformational stability of the enzyme. The extent of enzyme stabilization depends on the enzyme structure, enzyme immobilization methods, and the type of support. In this study, *Candida rugosa* lipase was successfully immobilized on PHBV by physical adsorption. Optimum conditions for CRL enzyme immobilization were as follows: 4 h immobilization time, immobilization temperature 25°C, and enzyme/support ratio of 0.3 (w/w) in hexane solvent. The stability of the immobilized CRL with respect to changes in pH and temperature, as well as the reusability and kinetic properties of the enzyme, were also improved versus the free enzyme, in agreement with the literature. Moreover, physicochemical analysis (FTIR, DSC, TGA, and NMR) showed that immobilization of CRL on PHBV by physical adsorption was effective. These results imply that PHBV is an excellent biocompatible support for enzyme immobilization.

Acknowledgments The authors acknowledge financial assistance from the Brazilian research funding agencies CAPES, CNPq, and FAPITEC/SE.

References

1. Amud AE, da Silva GRP, Tardioli PW, Soares CMF, Moraes FF, Zanin GM (2008) Methods and supports for immobilization and stabilization of cyclomaltodextrin glucanotransferase from thermoanaerobacter. Appl Biochem Biotechnol 146:189–201
2. Bryjak J, Trochimczuk AW (2006) Immobilization of lipase and penicillin acylase on hydrophobic acrylic carriers. Enzyme Microb Technol 39:573–578
3. Chen GQ, Wu Q (2005) The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials 26:6565–6578
4. ChiralVisão. http://www.chiralvision.com/immobead.htm. Accessed on May 2011
5. Costa MS, Duarte ARC, Cardoso MM, Duarte CMM (2007) Supercritical antisolvent precipitation of PHBV microparticles. Int J Pharm 328:72–77
6. Dandavate V, Keharia H, Madamwar D (2008) Ethy isovalerate synthesis using *Candida rugosa* lípase immobilized on sílica nanoparticles prepared in nonionic reverse micelles. Process Biochem 44:349–352
7. Fachin D, Loey AMV, Nguyen BL, Verlent I, Indrawati M, Hedricks ME (2002) Comparative study of the inactivation kinetics of pectinmethylesterase in tomato juice and purified form. Biotechnol Prog 18:739–744
8. Foresti ML, Ferreira ML (2007) Chitosan-immobilized lipases for the catalysis of fatty acid esterifications. Enzyme Microb Technol 40:769–777
9. Gonçalves SPC, Martins-Franchetti SM, Chinaglia DL (2009) Biodegradation of the films of PP, PHBV and its blend in soil. J Polym Environ 17:280–285
10. Guisan JM (2006) Immobilization and enzymes e cell, 2nd edn. Human, New Jersey
11. Huang XJ, Chen PC, Huang F, Ou Y, Chen MR, Xu ZK (2011) Immobilization of *Candida rugosa* lipase on electrospun cellulose nanofiber membrane. J Mol Catal B-Enzym 70:95–100
12. Karimpil JJ, Melo JS, D'Souza SF (2011) Hen egg white as a feeder protein for lipase immobilization. J Mol Catal B-Enzym 71:113–118
13. Li Y, Zhou G, Li C, Qin D, Qiao W, Chu B (2009) Adsorption and catalytic activity of Porcine pancreatic lipase on rod-like SBA-15 mesoporous material. Colloids Surf A Physicochem Eng Asp 341:79–85
14. Mateo C, Abian O, Fernández-Lafuente R, Guisán JM (2000) Increase in conformational stability of enzymes immobilized on epoxy-activated supports by favouring additional multipoint covalent attachment. Enzyme Microb Technol 26:509–515
15. Minakshi, Pundir CS (2008) Co-immobilization of lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase on to aryl amine glass beads affixed on plastic strip for determination of triglycerides in serum Indian. J Biochem Biophys 45(2): 111–115
16. Montero S, Blanco A, Virto DM, Landeta CL, Agud I, Solozabal R, Lascaray JS, Renobales M, Llama ML, Serra JL (1993) Immobilization of *Candida rugosa* lipase and some properties of the immobilized enzyme. Enzyme Microb Technol 15:239–247
17. Nasratun M, Said HA, Noraziah A, Abd Alla AN (2009) Immobilization of lipase from *Candida rugosa* on chitosan beads for transesterification reaction. Am J Appl Sci 6(9):1653–1657
18. Park EY, Sato M, Kojima S (2006) Fatty acid methyl ester production using lipase-immobilizing silica particles with different particle sizes and different specific surface areas. Enzyme Microb Technol 39:889–896
19. Persson M, Mladenoska I, Wehtje E, Adlercreutz P (2002) Preparation of lipases for use in organic solvents. Enzyme Microb Technol 31:833–841
20. Phadtare S, D'Britto V, Pundle A, Prabhune A, Sastry M (2004) Invertase-lipid biocomposite films: preparation, characterization, and enzymatic activity. Biotechnol Prog 20:156–161
21. Rodrigues RC, Bolivar JM, Palau-Ors A, Volpato G, Ayub MAS, Fernandez-Lafuente R, Guisan JM (2009) Positive effects of the multipoint covalent immobilization in the reactivation of partially inactivated derivatives of lipase from *Thermomyces lanuginosus*. Enzyme Microb Technol 44:386–393
22. Singh S, Mohanty AK (2007) Wood fiber reinforced bacterial bioplastic composites: fabrication and performance evaluation. Compos Sci Technol 67:1753–1763
23. Sivozhelezov V, Bruzzese D, Pastorino L, Pechkova E, Nicolin C (2009) Increase of catalytic activity of lipase towards olive oil by langmuir-film immobilization of lipase. Enzyme Microb Technol 44:72–76
24. Slater S, Mitsky TA, Houmiel KL, Hao M, Reiser SE, Taylor NB, Tran M, Valentin HE, Rodriguez DJ, Stone DA, Padgette SR, Kishore G, Gruys KJ (1999) Metabolic engineering of *Arabidopsis* and *Brassica* for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer production. Nature Am Inc 17:1011–1016
25. Soares CMF, De Castro HF, De Moraes FF, Zanin GM (1999) Characterization and utilization of *Candida rugosa* lipase immobilized on controlled pore silica. Appl Biochem Biotechnol 77–79:745–758
26. Soares CMF, Santana MHA, Zanin GM, De Castro HF (2003) Effect of polyethylene glycol and albumin in microbial lipase immobilization and in the catalysis in organic solution. Quím Nova 26:832–838 (in Portuguese)
27. Soares CMF, Dos Santos OAA, Olivo JE, De Castro HF, De Moraes FF, Zanin GM (2004) Influence of alkyl-substituted silane precursors of silica gels on the enzymatic activity of immobilized microbial lipase from *Candida rugosa*. J Mol Catal B-Enzym 29:69–79
28. Soares CMF, Santos OA, De Castro HF, Moraes FF, Zanin GM (2004) Studies on lipase immobilization in hydrophobic sol–gel matrix. Appl Biochem Biotechnol 113:307–319
29. Steinbuchel A (1996) PHB and other polyhydroxyalkanoic acids. In: Rehm HJ, Reed G, Roehr M (eds) Products of primary metabolism, vol 6, 2nd edn. Wiley, New Jersey, pp 405–464
30. Sun F, Sun Y, Yang H, Hou B (2009) Study of the lipase immobilized on PVA-alginate-active carbon. J Food Sci Biotechnol 4:4–12
31. Tutar H, Yilmaz E, Pehlivan E, Yilmaz M (2009) Inmobilization of *Candida rugosa* lípase on sporopollenin from *Lycopodium clavatum*. Int J Biol Macromol 45:315–320
32. Yadav GD, Jadhav SR (2005) Synthesis of reusable lipases by immobilization on hexagonal mesoporous silica and encapsulation in calcium alginate: transesterification in non-aqueous medium. Microporous Mesoporous Mater 86:215–222
33. Ye P, Xu K, Che AF, Wu J, Seta P (2005) Chitosan-tethered poly(acrylonitrile-co-maleic acid) hollow fiber membrane for lipase immobilization. Biomaterials 32:6394–6403
34. Yesiloglu Y (2005) Utilization of bentonite as a support material for immobilization of *Candida rugosa* lipase. Process Biochem 40:2155–2159
35. Zhu K, Jutila A, Tuominen EK, Patkar SA, Svendsen A, Kinnunen PK (2001) Impact of the tryptophan residues of *Humicola lanuginosa* lipase on its thermal stability. Biochem Biophys Acta 1547:329–338