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ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 51 (2008) 100–109
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Multipoint covalent immobilization of microbial lipase on chitosan and agarose activated by different methods

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Received 23 July 2007; received in revised form 30 October 2007; accepted 13 November 2007
Available online 19 November 2007

Abstract

In this work *Candida antarctica* lipase type B (CALB) was immobilized on agarose and chitosan. The influence of activation agents (glycidol, glutaraldehyde and epichlorohydrin) and immobilization time (5, 24 and 72 h) on hydrolytic activity, thermal and alkaline stabilities of the biocatalyst was evaluated. Protein concentration and enzymatic activity in the supernatant were determined during the immobilization process. More active derivatives were attained when the enzymatic extract was first purified through dialysis. The highest activities achieved were: for agarose-glyoxyl (with glycidol), 845 U/g of gel, after 72 h of immobilization; for chitosan-glutaraldehyde and agarose-glutaraldehyde, respectively, 1209 U/g of gel and 2716 U/g of gel, after 5 h of immobilization. Thermal stability was significantly increased, when compared to the soluble enzyme: 20-fold for agarose-glyoxyl (with glycidol)-CALB, 18-fold for chitosan-glutaraldehyde-CALB and 21-fold for agarose-glutaraldehyde. The best derivative, 58-fold more stable than the soluble enzyme, was obtained when CALB was immobilized on chitosan activated in two steps, using glycidol and glutaraldehyde, 72 h immobilization time. The stabilization degree of the derivative increased with the immobilization time, an indication that a multipoint covalent attachment between enzyme and the support had really occurred.

Keywords: *Candida antarctica* type B lipase; Chitosan; Enzyme immobilization; Enzyme stabilization

1. Introduction

In their natural environment, lipases (EC 3.1.1.3) catalyze the hydrolysis of esters formed from glycerol and long-chain fatty acids [1]. However, under appropriate experimental conditions, these enzymes are also very active biocatalysts for the esterification of fatty acids, alcoholysis and transesterifications reactions [2]. The activity of lipases is dramatically increased upon binding to the lipid surface, due to a conformational change of the enzyme. This is a distinctive feature from other esterases, which primarily hydrolyze water-soluble esters [3]. *Candida antarctica* lipase B (CALB) is preferred in many applications, because of its versatility with respect to substrates, high resistance to organic solvents, high thermal stability, stereo specificity and high enantioselectivity [4].

Enzyme immobilization on solid supports, besides facilitating the recovery and further re-use of the catalyst, offers important additional advantages. Immobilization avoids enzyme aggregation and autolysis, facilitates operational control, increases flexibility of reactor design and facilitates the removal from the reaction medium [5]. Yet, additional stabilization of the immobilized enzyme three-dimensional structure may be achieved if an increase in the rigidification of the macromolecule structure is promoted. This can be accomplished if several bonds between enzyme and support are obtained [6].

The multipoint covalent immobilization requires the interaction of several residues of the same enzyme molecule with active groups of the support. Enzyme stabilization is obtained after increasing the rigidity of a small part of its surface (e.g., 10–20%), which will turn the overall three-dimensional structure more rigid [6–9].

Aldehyde groups in the support and amine groups in the enzyme are a good choice to make the multipoint attachment and, therefore, to obtain highly stable enzyme derivatives. Amine

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doi:10.1016/j.molcatb.2007.11.016

groups (terminal and in lysine residues) are very reactive, abundant on the enzyme surface and form Schiff bases with the aldehyde groups of the support. The number of covalent bonds between the support and the enzyme depends on the degree of activation of the support (concentration of aldehyde groups in the support surface) and on the concentration of amine groups in the enzyme molecule [9,10]. pH is an important variable in this immobilization approach, since lysine amine groups have pK around 10.5, and will only be reactive above pH 10 [9,11].

Therefore, immobilization and stabilization of enzymes may make them still more attractive for industrial applications, facilitating their use under extreme conditions of temperature and pH, as well as in the presence of organic solvents or any other distorting agent. Improvement of stability, nonetheless, is still one of the main issues for the implementation of enzymes as industrial biocatalysts [12].

Due to the interesting properties of lipases, and specially CALB, as biocatalysts, several works report the immobilization of these enzymes using different protocols: adsorption on hydrophobic supports [13,14], entrapment in gels [15] and covalent attachment to solid supports [16–23]. Balcão et al. [2] and Paiva et al. [24] provide reviews of this theme.

It should be pointed out that lipases present a special characteristic, which brings about additional problems to the immobilization procedure. The active site of these enzymes is isolated from the reaction medium by a hydrophobic polypeptide chain. In the presence of hydrophobic drops of substrate, this lid opens and the active site is exposed. Hence, lipases are present in the reaction medium in two forms, the open and the close one [25]. Immobilization may decrease the enzyme activity, as it may prevent the interaction of the lid with external hydrophobic surfaces.

To overcome this problem, two approaches have been proposed: immobilization of the open form on hydrophobic supports [26] or immobilization in the presence of detergents to stabilize the open form [22]. Lipases also have been found to exhibit the tendency to form bimolecular aggregates, needing the presence of detergents during the immobilization procedure. Nevertheless, this phenomenon was not found to be significant for CALB [27], probably because this enzyme has a small lid [3,22]. In spite of these several studies on lipase immobilization, further investigations using different types of carriers are still necessary in order to improve the catalytic efficiency and to reduce the cost of the support.

Chitosan, an abundant raw material, has been already used as support for lipase immobilization [28]. This material is easily available in Ceará State, Brazil, due to the long extent of its seacoast and the high activity of its seafood industry [29]. It is a natural cationic polysaccharide derived from chitin and is known as a good support for enzyme immobilization because of its hydrophilicity, biocompatibility and biodegradability. Moreover, chitosan is obtained at a relatively low cost from shells of shellfish (mainly crab, shrimp, lobster and krill), wastes of the seafood industry [28] and its utilization for enzyme immobilization constitutes an attractive option for disposal of crustaceans, shrimp and crab shell wastes.

Chitosan has reactive amino and hydroxyl groups which after further chemical modifications, can make covalent bonds with reactive groups of the enzyme. Due to its amine groups, chitosan is a cationic polyelectrolyte (pK_a = 6.5) being insoluble in neutral aqueous solutions, but it is soluble in acidic solutions below pH 6.5. The mechanical properties of this polymer can be improved by further crosslinking using bifunctional reactants like glutaraldehyde [28]. Chitosan amine groups can directly react with glutaradehyde to generate aldehyde groups, which will in turn form Schiff bases with the enzyme [29]. Chytosan hydroxyl groups can be also activated by using epoxide reactants like glycidol and epichlorohydrin, for instance, followed by oxidation with sodium periodate to produce reactive aldehyde-glyoxyl groups [11].

Agarose gels, on their turn, are easily handled and activated due to presence of hydroxyl groups. They have a high surface area, an adequate porous diameter for protein immobilization, good mechanical properties [9] and have been successfully used for immobilization/stabilization of several enzymes [10,30], including lipases [21].

The only reactive groups in agarose are hydroxyls. Therefore, they can react with epoxide reagents to generate glyoxyl groups, in the same way as chitosan hydroxyl groups do. These aldehyde groups are less reactive than the ones from glutaraldehyde. While the latter can immobilize the enzyme after linking with only one amine group, the former one will only able to keep the enzyme linked if at least two bonds are formed. Using glutaraldehyde, the linkage can be done using the terminal amine group of the protein while with glyoxil groups the ε-amino groups of the lysine residues have to be available to make feasible the immobilization. The higher the concentration of amine groups in the enzyme and of glyoxyl groups in the support, more bonds can occur. The requirement of high concentrations of amine groups gives glyoxyl an additional advantage with respect to glutaraldehyde: the immobilization using glyoxyl groups has to proceed via the area(s) of the protein that have more reactive residues, enabling intense multipoint enzyme–support interaction.

However, glyoxyl groups are only able to immobilize proteins at pH above 10 [11], because the pK_a of lysine residues is 10.5. On the other hand, the high reactivity of the aldehyde groups of glutaraldehyde makes it very versatile, being used in very different conditions. The usual pH to perform the immobilization on supports activated with glutaraldehyde is around 7–8.5, because these aldehyde groups are more stable in this pH range. However, at these more neutral pH values, the reactivity of ε-amino of Lys groups may be expected to be quite low and the intensity of the multipoint covalent attachment may be not very high [12]. The activation of chitosan with glutaraldehyde is easy, since chitosan already has amine groups that can be directly activated. Agarose-glutaraldehyde gels have to be prepared reacting ethylenediamine with a previously prepared agarose-glyoxyl support [31]. It has been also shown that this treatment permits to have all the generated amino groups covered by a dimer of glutaraldehyde [32].

The enzyme–support interactions formed during the immobilization process can modify the conformational structure, resulting in a modulation of the enzyme catalytic properties. Dif-

ferent factors may influence the immobilization process, such as: activating agents, nature of the support and degree of interaction between enzymes and support [6,33]. The aim of this work was to study the influence of three different activating agents on CALB activity and stability after immobilization on chitosan and agarose.

2. Materials and methods

2.1. Materials

Native soluble lipase B from *C. antarctica* B (Lipozyme® CALB L) was kindly donated by Novozymes Latin America Ltd. (Araucária, Brazil). Agarose gels CL 6% were obtained from Amershan Bioscience (Uppsala/SU) and powdered chitosan, 85.2% deacetylation degree was purchased from Polymar Ind Ltda (Ceará, Br). Glycidol, epichlorohydrin, methyl butyrate and bovine serum albumin were acquired from Sigma–Aldrich Chemical Co. (St. Louis, MO). Glutaraldehyde was from Vetec (São Paulo, Br).

2.2. Assay of hydrolytic activity: methyl butyrate hydrolysis

Hydrolytic activity was determined by methyl butyrate hydrolysis. Experiments were performed using an automatic titrator (pHstat), using 50 mM NaOH solution as titrating agent [34]. The pH was set at 7.0 and the temperature was 28 °C. In this work, one unit (U) of enzymatic activity was defined of the amount of enzyme that hydrolyses 1 μmol of methyl butyrate per minute at pH 7.0 and 28 °C.

2.3. Protein concentration

Protein concentration was measured according to Bradford [35] using bovine serum albumin (BSA) as a standard.

2.4. Support preparation and activation

2.4.1. Preparation of chitosan beads

Four grams of powdered chitosan was added to 96.0 mL of a 5% acetic acid solution. The obtained solution was dropped into a gently stirred 1 M NaOH solution for 24 h, at room temperature. Afterwards, they were washed with an excess of distilled water. Agarose beads were obtained from Amershan Bioscience.

2.4.2. Activation with glycidol and epichlorohydrin

Chitosan-diol and agarose-diol were prepared by mixing beads under stirring with an aqueous solution containing NaOH 1.7 M and $NaBH_4$ 0.75 M (glycidol) [9] or NaOH 2 M and $NaBH_4$ 0.12 M (epichlorohydrin) [36] at ice bath. Then, 0.48 mL of glycidol or 1 mL of epichlorohydrin per gram of bead were added and kept under mechanical stirring for 18 h and washed until neutrality.

2.4.3. Preparation of glyoxyl-supports

Chitosan-diol beads and agarose-diol beads were contacted with 1 mL of 0.1 M $NaIO_4$ solution per gram of gel for 2 h at room temperature [9]. Afterwards, they were washed with an excess of distilled water until neutrality. Analysis of oxirane groups was performed according to Sundberg and Porath, 1974 [37] with slightly modifications. The release of hydroxyl groups was followed by titration with 0.1 M hydrochloric acid. Oxirane in solution was determined as follows. The oxirane-containing gel (100 mg) was added to 15 mL of 1.3 M sodium thiosulphate solution and pH was kept constant (pH 7.0) by addition of hydrochloric acid until the reaction was complete. The amount of epoxide present in solution was then calculated from the amount of hydrochloric acid needed in order to maintain neutrality.

2.4.4. Preparation of agarose-glutaraldehyde

This support was prepared from agarose-glyoxyl beads [31], by adding 10 g of these beads to a 40 mL of a 2 M ethilenediamine (EDA) solution at pH 10. Afterwards, 11.2 mL of a 0.2 M phosphate buffer solution (pH 7.0) and 16.8 mL of a 25% glutaraldehyde solution were added to the glyoxyl-amine support and the system was kept under gentle stirring for 16 h at room temperature. Finally, the activated support was washed with water and vacuum dried.

2.4.5. Preparation of chitosan-glutaraldehyde

Glutaraldehyde activation was made by contacting chitosan beads with sodium phosphate buffer 0.1 M, pH 7.0 containing glutaraldehyde 5% (v/v) using a ratio V_{beads}/V_{total} of 1/10 during 1 h at 25 °C. Afterwards, the beads were washed with distilled water to remove the excess of the activating agent.

2.4.6. Reaction of the remaining chitosan amine groups with different aldehydes

One gram of chitosan-diol was added to 1 mL of 20% formaldehyde, glyoxal or glutaraldehyde solution. The reaction mixture was gently stirred at 25 °C for 2 h [38]. Finally, oxidation with periodate was carried out and the support was used to CALB immobilization, according to the previously described conditions.

2.5. Determination of the aldehyde content on agarose and chitosan

Aldehyde content on agarose-glyoxyl$_{Glyc}$ was determined by quantification of the amount of sodium periodate consumed during oxidation [9]. However, aldehyde content on chitosan supports and agarose activated using glutaraldehyde or epichlorohydrin was estimated by Schiff reaction method. Ten milligrams of support were added to 1 mL of Schiff reagent and kept in rest for 2 h. Afterwards, the suspension was diluted and centrifugated for 3 min at 5000 rpm being the absorbance measured at 450 nm and aldehyde concentration calculated trough a previous calibration curve by using agarose-glyoxyl$_{Glyc}$ with different aldehyde amounts as reference.

2.6. Immobilization of CALB

CALB was immobilized on agarose and chitosan gels, after activation with glycidol, epichlorohydrin or glutaraldehyde. The

immobilization was carried out in 100 mM bicarbonate buffer, pH 10.05, at 25 °C and different incubation times (5, 24 and 72 h), under gentle stirring. After immobilization, sodium borohydride (0.5 mg/mL of reaction medium) was added to system that was kept at ice bath. In most essays, 3 mg of protein per gram of support was used for immobilization, prepared from a crude extract containing 17.8 mg of protein per milliliter, except when studying the effect of the activation method on the amount of immobilized enzyme. In this case, the amount of protein immobilized per gram of support varied from 1.5 to 60 mg/g support and a dialyzed enzyme extract was used, containing 6.2 mg of protein per milliliter.

2.7. Thermal stability essays

Samples of free or immobilized CALB were incubated in 25 mM sodium phosphate buffer, pH 7 at 50 °C. Periodically, samples were withdrawn and their residual activities were assayed. The deactivation constant and half-life ($t_{1/2}$) for each immobilized derivative was calculated according to the model proposed by Sadana and Henley [39].

2.8. Alkaline stability essays

Alkaline stability was investigated by incubation of samples in an alkaline solution, pH 12 at 25 °C. Samples were withdrawn at selected time intervals and residual activities were determined.

2.9. Dialysis

CALB crude extract, 5 mL, was dialyzed using a 14 kDa cellulose membrane and 500 mL of a 5 mM phosphate buffer solution, pH 7, at 4 °C, during 24 h. The 500 mL of buffer solution was replaced each 8 h.

2.10. Immobilization parameters

Immobilization yield (IY) was calculated by measuring the difference between protein concentration on the supernatant before (Ct_0) and after (Ct_t) immobilization, according to Eq. (1):

$$IY\% = \frac{Ct_0 - Ct_t}{Ct_0} \times 100 \quad (1)$$

In this work, recovered activity is defined as the ratio of apparent hydrolytic activity, obtained by the direct measure of derivative activity, and the difference between hydrolytic activity on the supernatant before and after immobilization, which can be considered as the derivative theoretical activity. Therefore, recovered activity can be considered as the biocatalyst effectiveness.

3. Results and discussion

3.1. Influence of activation method and incubation time on immobilization yield and recovered activity

Table 1 shows the results of the degree of activation for chitosan and agarose activated with glycidol, epichlorohydrin and glutaraldehyde. It can be seen that different concentrations of aldehyde groups were obtained depending on the support and the activating reactant. Supports activated with epichlorohydrin displayed higher degree of activation than the ones activated with glycidol. Epichorohydrin is more reactive than glycidol, and was present in higher concentration.

The high concentration of amine groups in chitosan (degree of deacetilation, 85.2%) also explains the high degree of activation obtained with glutaraldehyde, because it can react directly with the amine groups of chitosan. However, chitosan-glyoxyl-glutaraldehyde shows low degree of activation. This result may be explained by the previous reaction with glycidol, which can react easier with amine than with hydroxyl groups. Therefore, amine groups of chitosan are transformed in hydroxyl ones and, very likely, the spacer arm generated would be different. Oxidation with sodium periodate of this amino-diol may be also more complex. If this is the case, the amine groups of chitosan would be transformed in amino-diols and the oxidation reaction conditions might not be appropriate to generate aldehyde groups.

Table 2 shows the results of recovered activity ($A_{Rec.}$), immobilization yield (IY) and derivative activity (A_{Der}), for the different immobilization methods and supports studied in this work. All prepared derivatives were obtained by immobilizing the enzyme at pH 10.05. Although glutaraldehyde is reported to be not very stable in this condition [12], it was nonetheless used, so that the supports would be compared using the same immobilization conditions. Furthermore, from the results presented in Table 2, this phenomenon did not seem to be very important for

Table 1
Support moisture (%) and epoxy and aldehyde group concentration of chitosan and agarose after activation with glycidol, epichlorohydrin and glutaraldehyde

Activated support	Support moisture (%)	Aldehyde groups concentration (μmol/g dried support)	Epoxy concentration (μmol/g wet support)
Agarose-glyoxyl$_{glyc}$	90.89	878 (80)[a]	–
Chitosan-glyoxyl$_{glyc}$	94.47	687 (38)[a]	–
Agarose-glutaraldehyde	88.26	605 (71)[a]	–
Chitosan-glutaraldehyde	87.47	1692 (212)[a]	–
Agarose-glyoxyl$_{epi}$	91.94	1327 (107)[a]	6.5 ± 0.3
Chitosan-glyoxyl$_{epi}$	89.32	1835 (196)[a]	43.0 ± 4.0
Chitosan-(glyoxyl[a]/glutaraldehyde)	91.84	621 (50.7)[a]	–

[a] Aldehyde groups concentration/g wet support.

Table 2
Effect of immobilization time on yield immobilization (IY) and recovered activity ($A_{Rec.}$) of CALB immobilized derivatives

Immobilized derivative	5[a]			24[a]			72[a]		
	IY (%)	A_{Der} (U/g)	$A_{Rec.}$ (%)	IY (%)	A_{Der} (U/g)	$A_{Rec.}$ (%)	IY (%)	A_{Der} (U/g)	$A_{Rec.}$ (%)
Agarose-glyoxyl$_{glyc}$[b]	63	180	81	72	214	71	75	144	86
Chitosan-glyoxyl$_{glyc}$[b]	5	8	5	37	13	12	38	16	15
Agarose-glutaraldehyde	89	330	68	92	300	62	92	300	61
Chitosan-glutaraldehyde	90	402	82	90	295	58	78	306	59
Agarose-glyoxyl$_{epi}$[c]	58	106	57	73	157	68	73	130.7	24
Chitosan-glyoxyl$_{epi}$[c]	5	23	38	15	45	63	17	44.8	36

$A_{Rec.} = A_{Der}/At_{Sup}$ (disappeared on supernatant). A_{Der} is activity measured on the immobilized derivative; IY percentage of protein disappeared on supernatant. Enzyme loading offered: 3 mg protein/g support.
[a] Incubation time (h).
[b] Glyoxil groups obtained from glycidol.
[c] Glyoxil groups obtained from epichlorohydrin.

lipase immobilization, because the best results of immobilization yield and hydrolytic activities of the immobilized enzyme were reached when glutaraldehyde was used as activating agent: IY of 89% and A_{Der} of 330 U/g for agarose-glutaraldehyde; and IY of 90% and A_{Der} 402 U/g for chitosan-glutaraldehyde, with enzyme load of 3 mg/g support. Similar results were obtained by [21] with immobilized CALB, used for resolution of cyclopyrrolone compounds.

It can be observed from the results showed in Table 2 that immobilization yields and activities of chitosan-glyoxyl derivatives are much lower than the ones of agarose-glyoxyl derivatives. The lower concentration of aldehyde groups for chitosan activated with glycidol, when compared with agarose activated with glycidol, may explain these results. However, when epichlorohydrin was used, the degree of activation was higher for chitosan-glyoxyl$_{epi}$ than for agarose-glyoxyl$_{epi}$. Therefore, another phenomenon may be preventing the formation of bonds between enzyme and aldehyde groups of chitosan-glyoxyl$_{epi}$. A possible explanation is a decrease in the porous diameter of chitosan after activation with epichlorohydrin. If that occurred, despite the high aldehyde concentration the enzyme would not reach most of these groups. It was also observed during the immobilization procedure that after activation of chitosan with epichlorohydrin the support lost a great amount of water, which may indicate a significant increasing in its hydrophobicity. The moisture of this support, as showed in Table 1, corroborates this hypothesis. Therefore, physical changes in the support surface may not favor the immobilization of CALB and could explain the poor results for chitosan-glyoxyl obtained via epichlorohydrin activation. With respect to the influence of the immobilization time on the immobilization parameters, it can be observed that, except for chitosan-glyoxyl, there was no significant increasing in the immobilization yields after 5 h of process. At alkaline pH, protein was immobilized within only a few minutes in highly activated supports. The first bonds between enzyme and activated support, one for aldehydes from glutaraldehyde, and at least two for glyoxyl groups, are built up quickly. However, the next bonds will take more time to be formed as the support surface and the protein are not complementary structures. Thus, after the first linkages, enzyme loses flexibility and new bonds are more difficult to be established, needing longer incubation times to allow the correct alignment between the groups placed in enzyme and support [11]. Therefore, longer incubation time would imply more enzyme–support links. In consequence, a decrease in enzyme activity due to the conformational changes and a more rigid enzyme structure would be expected. The decrease in activities of the immobilized enzyme after 24 h, observed in the results of Table 2, could be due to these conformational changes. On the other hand, an increase in thermal and alkaline stabilities of the derivatives was expected as well.

3.2. Effect of incubation time on the thermal stability of immobilized CALB

Fig. 1 shows the residual activity of different immobilized derivatives incubated at 50 °C for 72 h. Best results of thermal stabilities were obtained when CALB was immobilized on agarose-glutaraldehyde and agarose-glyoxyl$_{glyc}$, which retained around 30% of the initial activity after 24 h of incubation. The

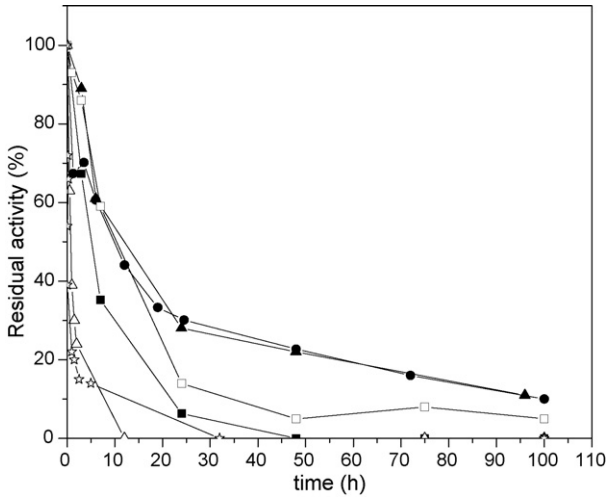

Fig. 1. Thermal inactivation profile at 50 °C of soluble (★) and immobilized CALB, obtained after 72 h of incubation with different supports: (●) agarose-glutaraldehyde; (■) agarose-epichlorohydrin; (▲) agarose-glycidol; (□) chitosan-glutaraldehyde; (△) chitosan-epichlorohydrin. Enzyme load: 3 mg of protein/g support.

Table 3
Influence of the immobilization time on the thermal stabilities of prepared CALB derivatives

Immobilized derivatives	$t_{1/2}$ (h) for different incubation times		
	5 h	24 h	72 h
Agarose-glyoxyl$_{glyc}$	3.7	5.5	10.2
Chitosan-glyoxyl$_{glyc}$	1.8	1.4	–
Agarose-glutaraldehyde	4.3	6.3	9.0
Chitosan-glutaraldehyde	4.9	5.4	10.6
Agarose-glyoxyl$_{epi}$	2.9	3.9	4.8
Chitosan-glyoxyl$_{epi}$	0.4	0.2	0.8

Half-lives ($t_{1/2}$) determined at 50 °C, pH 7.0. Soluble CALB presented a half-life of 0.5 h. Enzyme load: 3 mg protein/g support.

thermal inactivation curves of these derivatives show similar profiles, which suggest the presence of sub-populations of enzymes that are immobilized on the support through the binding of two or more residues [40]. Arroyo et al. [17] have obtained similar stabilization of CALB immobilized on different supports. They have observed that the biocatalyst retained 30–60% of its initial activity after 24 h of incubation in wet conditions at 50 °C, depending on the support used.

Table 3 shows the values of half-life determined for derivatives prepared using different immobilization times. These values were calculated from experimental data of thermal stability at 50 °C, using the parameters of a thermal inactivation model proposed by Sadana and Henley [39].

Comparing the data presented on Table 3, it can be observed that all derivatives obtained after 72 h of immobilization showed higher half-lives than the ones obtained after 24 h, except for chitosan-glycidol. These results suggest that multipoint covalent attachment has occurred for these derivatives, since the protein concentration measured in the supernatant was constant after 24 h of immobilization (data not shown). As already discussed, when multipoint immobilization occurs, although the first binding is fast, the multi-interaction process requires longer times to achieve the correct alignment of the reactive groups of enzyme and support [11]. Therefore, the fact that a higher stabilization was achieved after longer incubation times indicates that CALB is attached to the support by more than one bond.

3.3. Effect of activation method on the alkaline stability of CALB derivatives

According to [4], soluble CALB presents maximum activity at pH 7.0 with rapid inactivation bellow pH 6.0 and above pH 8.0. Therefore, alkaline stability was investigated by incubation of samples at pH 12 and 25 °C and results are in Fig. 2.

The stability of the three-dimensional structure submitted to treatment with different distorting agents (temperature, extreme pH) is an indirect form to confirm the formation of multipoint covalent attachment [40]. It can be observed in Fig. 2 that all derivatives, except chitosan-epichlorohydrin, are more stable than soluble CALB. Chitosan-epichlorohydrin was only 1.6-fold more thermal stable than the soluble enzyme (see half lives on Table 3), which indicates that the immobilization of CALB on

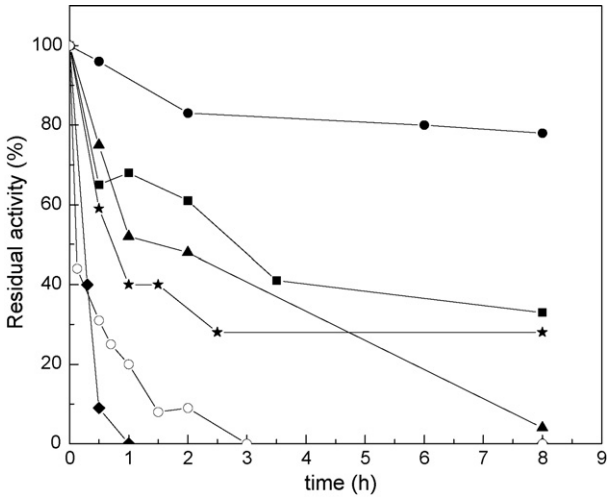

Fig. 2. Alkaline inactivation profiles at pH 12 and 25 °C of soluble (◯) and immobilized CALB, obtained after 72 h of incubation with different supports: (●) agarose-glutaraldehyde; (■) agarose-glycidol; (▲) agarose-epichlorohydrin; (♦) chitosan-epichlorohydrin; (★) chitosan-glutaraldehyde. Enzyme load: 3 mg of protein/g support.

chitosan-epichlorohydrin is not a result of a multipoint covalent attachment.

Although excesses of glutaraldehyde and epichlorohydrin have been used, the different experimental conditions, and the chemical reactivities of the activation agents promoted the formation of supports with different degrees of activation. In chitosan, the presence of strongly reactive amine groups suggests that several parallel reactions might proceed simultaneously. In an attempt to have a better understanding of these reactions and to improve the immobilization protocol, a different approach for the activation of chitosan was studied.

3.4. Effect on CALB immobilization of the reaction of the remaining chitosan amine groups with different aldehydes

Chitosan was first activated with glycidol or epichlorohydrin to form glyceryl groups. Glycidol, epoxy-propanol, has epoxy and hydroxyl groups while epichlorohydrin has epoxy and chloride groups, being the latter one more reactive. As the support amine groups are more reactive than the hydroxyl ones, after the reaction with glycidol or epichlorohydrin, only the remaining amine groups in chitosan will be able to react with the aldehydes, in the next step. Although it has been reported that only few chitosan amine groups reacts with epichlorohydrin, the reaction conditions used in this work were stronger and it was expected that more amine groups were transformed into amino-diol [41]. After the reaction with glycidol or epichlorohydrin, the support was treated with an aldehyde solution containing glutaraldehyde, formaldehyde or glyoxal. Table 4 shows the experimental conditions and values of hydrolytic activity obtained using the described activation protocol.

It can be observed that the reaction of chitosan amine groups with formaldehyde (entry 2) or glyoxal (entry 3) was not efficient to increase the activity of CALB derivatives, when comparing to the derivatives prepared using only the activation with glyci-

Table 4
Influence of the reaction of the amine groups of chitosan with the aldehydes formaldehyde, glyoxal or glutaraldehyde on the immobilization parameters and half-life ($t_{1/2}$) of the several CALB-chitosan derivatives

Entry	CALB-chitosan derivative-activation agents	Parameters			
		IY (%)	A_{Der} (U/g)	$A_{Rec.}$ (%)	$t_{1/2}$ (h)
1	Only glycidol	5	8	5	1.8
2	Glycidol-formaldehyde	18	3	3	–
3	Glycidol-glyoxal	8	7	4	–
4	Glycidol-glutaraldehyde	73	245	62	12.6
5	Epichlorohydrin-glutaraldehyde	60	256	100	5.4
6	Only epichlorohydrin	5	23	38	0.4
7	Only glutaraldehyde	90	402	82	4.9

Immobilization time: 5 h. Enzyme load: 3 mg protein/g support. Half-life ($t_{1/2}$) of soluble CALB = 0.5 h.

dol (entries 1 and 6). The chemistry of the reaction with these aldehydes is very complex and the several side reactions that are reported to occur may explain the obtained results. Even formaldehyde, a mono functional reagent, is reported to be able to act as a cross linking agent [42]. The treatment of chitosan-diol with formaldehyde and glyoxal was carried out using experimental conditions in which no reaction would occur with the hydroxyl groups and only amine groups would be consumed [38]. Although poor results have been obtained after the reaction with glyoxal and formaldehyde, when the amine groups reacted with glutaraldehyde (see entry 4 of Table 4), the derivative was 30-fold more active than chitosan-glycidol (entry 1), while the derivative in entry 5 was 11-fold more active than chitosan-epichlorohydrin (entry 6).

Higher thermal stability was also observed for CALB immobilized on chitosan-glycidol-glutaraldehyde ($t_{1/2}$ = 12.6 h) when compared to CALB immobilized on chitosan-glycidol ($t_{1/2}$ = 1.8 h) and on chitosan-glutaraldehyde ($t_{1/2}$ = 4.9 h).

The results obtained here may be explained by the differences on physical properties observed when chitosan is treated with glutaraldehyde, formaldehyde or glyoxal [42]. Different degrees of crosslinking are obtained with the different aldehydes, which would lead to different porosities and different water retention capacities of the support. Moreover, when two activation agents are used simultaneously, multipoint immobilization is favored and higher thermal stability is observed. On the other hand, in some areas of the support surface, this high activation degree might cause conformational changes in the enzyme structure and consequently cause loss of activity. The high degree of crosslinking might also hinder the entrance of enzyme within the pores of the support. Besides, the decrease of the mean porous radius of the support may enhance intraparticle diffusion effects, decreasing the apparent activity of the immobilized enzyme (recovered activity).

Nevertheless, an important improvement in the immobilization protocol using chitosan as support was obtained with these experiments. The production of glyceril groups in chitosan, followed by activation of the support with glutaraldehyde and, finally, oxidation with sodium periodate to generate glyoxyl groups seems to result in a good activated support for CALB immobilization. Immobilization time was confirmed to be important to improve the stability of the CALB derivatives. Initially, a half-life of 12.6 h was achieved for chitosan-glyoxyl$_{glyc}$-glutaraldehyde-CALB using only 5 h of immobilization time—already higher than the highest one obtained for the previous prepared CALB derivatives (Table 2). The chitosan-glyoxyl$_{glyc}$-glutaraldehyde support was then contacted with CALB for longer immobilization times. The half-life of the derivative obtained after 72 h reached a half-life of 29 h, at 50 °C, which means a stabilization factor of 58 with respect to the soluble enzyme. Arroyo et al. [17] immobilized covalently CALB on agarose, alumina and silica. Their best result, obtained by immobilization of CALB on silica activated with 2,4,6-trichlorotriazine, was a stabilization factor of 48, at 50 °C, for the hydrolysis of triacetin. Novozym 435, a commercial immobilized CALB presented a stabilization factor of 28, at the same experimental conditions. The stabilization factor obtained in this work, 58, is higher, but it is still much lower than the ones reported for other immobilized enzymes [10,30]. It seems not to be easy to achieve very high stabilization factors for CALB, which is reported to have 317 residues and only nine are lysines [3]. This low number of lysines may explain the lower stabilization factor when compared to the ones obtained for other enzymes. Besides, the half-life of the soluble enzyme is 30 min at 50 °C, a quite high figure, indicating that the soluble enzyme may already possess a more rigid structure.

3.5. Effect of the reduction with sodium borohydride on the activity of immobilized CALB

Although support activation is necessary to immobilize enzymes, it is important that only the reactive groups of the enzyme can catalyze reactions under industrial conditions, that is, the support must be inert after enzyme immobilization. Also, undesired enzyme–support interactions may be caused by non-inert support surfaces, causing the decrease of enzyme activity and stability [43,44]. The use of reducing agents, e.g., sodium borohydride, turns the produced derivative suitable to use in drastic reaction conditions (temperature, pH and organic solvent). Sodium borohydride is a double-functional agent that is able to inactivate the remaining aldehydes groups present in the support after immobilization [45,46]. The final reduction of the immobilized enzyme with sodium borohydride transforms reversible Schiff bases into stable secondary amino bounds, and the remaining aldehydes on the solid support become hydroxyl groups, which are much less reactive than aldehyde.

Table 5
Influence of $NaBH_4$ concentration and temperature on the activity of agarose-glyoxyl–CALB

Experimental reduction condition	A_{Der} (U/g)	Residual activity (%)
Unreduced	350 ± 10	100
0.5 mg mL^{-1} (4 °C)	355 ± 30	100
0.5 mg mL^{-1} (25 °C)	238 ± 3	68
1.0 mg mL^{-1} (4 °C)	289 ± 0	83
1.0 mg mL^{-1} (25 °C)	240 ± 12	69

However, some enzymes might lose catalytic activity by reduction of disulfide bonds when the derivatives are submitted to treatment with sodium borohydride ($NaBH_4$) under drastic reaction conditions [46]. Therefore, it is important to study the reduction step in order to define an adequate immobilization protocol. Different reduction conditions using sodium borohydride were investigated, aiming at preparing stable immobilized derivatives, and also avoiding significant loss of catalytic activity. Table 5 shows the results of derivative activity and residual activity for different $NaBH_4$ concentrations (0.5 and 1.0 mg mL^{-1}) and temperatures (25 and 4 °C).

It can be observed that the higher the temperature and the concentration of $NaBH_4$, the lower is the residual activity of the immobilized enzyme. The derivative only remained fully active after the reduction step when the process was conducted at 4 °C and using the lowest $NABH_4$ concentration (0.5 mg mL^{-1}). Therefore, this condition was selected for further studies.

Other authors [45,47] have also reported loss of derivative activity after reduction with $NaBH_4$. Deleterious effects of sodium borohydride on protein structures were observed: disulfide bond splitting and reductive cleavage of peptide bonds [46]. These effetcs could justify the poor results obtained at 25 °C with 1.0 mg mL^{-1} of $NaBH_4$.

3.6. Effect of the activation method on the amount of immobilized enzyme

The effect of enzyme load on the biocatalyst properties was first investigated using agarose activated with glycidol. Two protein concentrations were offered for immobilization, 5 and 10 mg of protein/g support. The lower enzyme load led to a derivative activity of 300 U/g. When 10 mg of enzyme/g support were offered, the derivative activity was 276 U/g, but the amount of immobilized protein was twofold higher. There are two possible explanations for these results. First, the crude enzymatic extract may contain impurities, which compete for the reactive groups of the support. Moreover, the immobilized enzyme may become a barrier to products and substrates diffusion.

The presence of smaller proteins (around 12 and 30 kDa) in the crude extract of CALB was already observed [48]. The crude extract was then submitted to dialysis, aiming to separate the desired enzyme from these impurities. After the purification step, CALB derivatives with 845 U/g of activity were obtained (for 60 mg of enzyme/g support). Therefore, the removal of crude extract impurities was very important since the immobilization method is not selective and smaller proteins or peptides

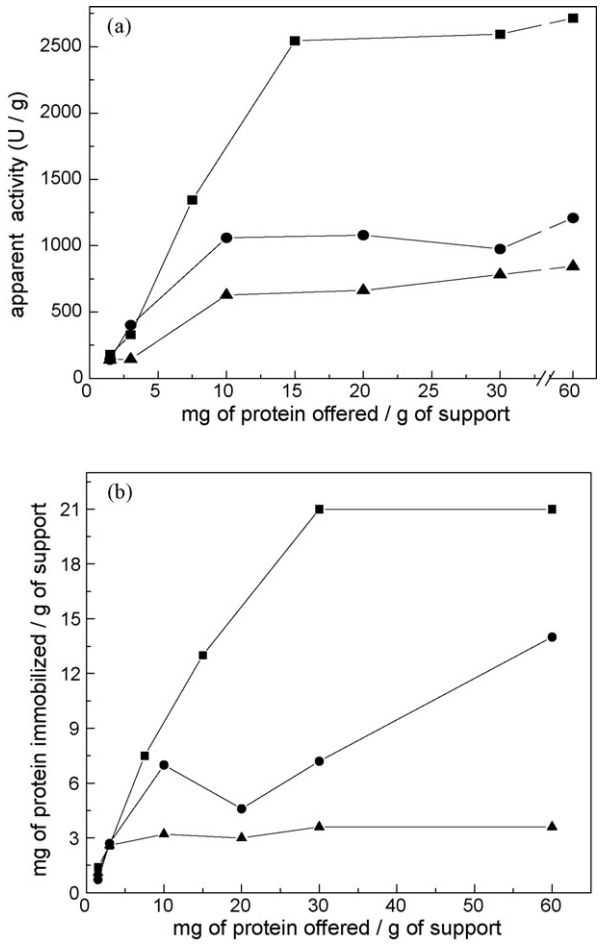

Fig. 3. Effect of enzyme load in (a) CALB apparent activity and (b) amount of immobilized protein: (●) chitosan-glutaraldehyde; (■) agarose-glutaraldehyde; (▲) agarose-glycidol.

(contaminants) diffuse faster and consume preferentially the reactive groups on the surface support [9].

The supports agarose-glycidol (72 h of incubation), agarose-glutaraldehyde and chitosan-glutaraldehyde (5 h of incubation) showed the best results as a trade-off between derivative activity and stability. Therefore, they were selected for further studies using high enzyme loads. Fig. 3a and b shows, respectively, the derivative apparent activity and the amount of protein attached per gram of support. Table 6 shows results of recovered activities, derivative apparent activity and half-lives for the several prepared derivatives. The thermal stability assays for the derivatives prepared with several enzyme loads were conducted in order to check the presence of mass transfer effects.

It can be observed that the high reactivity of glutaraldehyde led to CALB derivatives with higher activity using both chitosan and agarose supports. When agarose-glutaraldehyde was used for CALB immobilization, the best results of amount of immobilized protein (∼20 mg of protein/g support) and apparent activity (∼2500 U/g support) were obtained.

An increase of stability as enzyme load increases could be observed when CALB was immobilized on supports activated with glutaraldehyde. This trend confirms that the obtained stability results when high enzyme loads are immobilized might be

Table 6
Immobilization parameters for several CALB derivatives obtained with different enzyme loads: disappeared protein in supernatant (IY), activity of derivative (A_{Der}), recovered activity ($A_{Rec.}$) and half-life ($t_{1/2}$) at 50 °C

Enzyme load (mg/g support)	Parameter	CALB derivatives (immobilization time)			
		Agarose-G[a] (72 h)	Agarose-GA[b] (5 h)	Chitosan-GA (5 h)	Chitosan-G-GA[c] (5 h)
1.5	$A_{Rec.}$ (%)	81	99	80	–
	$t_{1/2}$	1.2	2.4	0.4	–
	A_{Der} (U/g)	140	179	141	–
3.0	$A_{Rec.}$ (%)	86	68	82	62
	$t_{1/2}$	10.2	4.3	4.9	12.6
	A_{Der} (U/g)	144	330	402	245
15.0	$A_{Rec.}$ (%)	90	62	89	–
	$t_{1/2}$	4.0	4.8	4.2	–
	A_{Der} (U/g)	170	2545	336	–
30.0	$A_{Rec.}$ (%)	100	49	39	–
	$t_{1/2}$	7.9	10.6	5.2	–
	A_{Der} (U/g)	782	2303	976	–
60.0	$A_{Rec.}$ (%)	100	29	48	27
	$t_{1/2}$	8.7	10.6	3.4	29
	A_{Der} (U/g)	845	2716	1209	346

[a] G, glycidol.
[b] GA, glutaraldehyde.
[c] G-GA, glycidol and glutaraldehyde.

somehow masked. Indeed, Thiele number increases with reaction rates, leading to smaller effectiveness factors. Therefore, the thermal stability results obtained using a high amount of immobilized enzyme may be only apparent, and that fact should be considered when evaluating the effect of different immobilization strategies on the derivative stability [49].

If the inherent reaction rate that corresponds to the enzyme activity is higher than the rate of substrate diffusion, i.e. the rate-controlling step is mass transport in the pores, the apparent enzymatic activity of high load derivatives is lower than the inherent one (without mass transfer effects). In other words, when determining thermal stability, the first enzyme molecules that are inactivated are replaced by other ones that were not contributing before for the measured reaction rate (due to the mass transfer delay), and the biocatalyst becomes apparently more stable. Therefore, in order to accurate estimate thermal stabilities, it is convenient to use derivatives with low enzyme load or to use a substrate that has low inherent reaction rates. This is not the case of this work, since butyl butyrate hydrolysis catalyzed by CALB is a fast reaction.

4. Conclusions

The use of different activation agents allowed the production of CALB derivatives with different properties. Agarose activated with glutaraldehyde led to the highest derivative activity, 2700 U/g and half-life of 10.6 h. The best derivative, 58-fold more stable than the soluble enzyme, was obtained by immobilizing CALB on chitosan activated in two steps, using glycidol and glutaraldehyde. The increase of immobilization time enhanced the thermal stability of the derivative, a strong indication that multipoint immobilization has occurred. However, the longer the immobilization time, the lower the derivative activity. Therefore, the immobilization time that should be chosen to produce the derivative is a trade-off between these two parameters.

Acknowledgments

The authors would like to thank the Brazilian research-funding agencies FUNCAP (State of Ceará), FAPESP (State of São Paulo), and CNPq, FINEP and CAPES (Federal).

References

[1] R. Sharma, Y. Chisti, U.C. Banerjee, Biotechnol. Adv. 19 (2001) 627–662.
[2] V.M. Balcão, A.L. Paiva, F.X. Malcata, Enzyme Microb. Technol. 18 (1996) 392–416.
[3] J. Uppenberg, M.T. Hansen, S. Patkar, T.A. Jones, Structure 2 (1994) 293–308.
[4] R.W. McCabe, A. Rodger, A. Taylor, Enzyme Microb. Technol. 36 (2005) 70–74.
[5] J.M. Guisan, Immobilization of Enzymes and Cells, 2nd ed., Humana Press Inc., Totowa, N.J., 2006.
[6] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Enzyme Microb. Technol. 40 (2007) 1451–1463.
[7] A.M. Klibanov, Adv. Appl. Microbiol. 29 (1982) 1–28.
[8] V.V. Mozhaev, V.A. Siksnis, Biotechnol. Bioeng. 25 (1983) 1937–1945.
[9] J.M. Guisan, Enzyme Microb. Technol. 10 (1988) 375–382.
[10] J. Pedroche, M.M. Yust, C. Mateo, R. Fernández-Lafuente, J. Girón-Calle, M. Alaiz, J. Vioque, J.M. Guisán, F. Millán, Enzyme Microb. Technol. 40 (2007) 1160–1166.
[11] C. Mateo, J.M. Palomo, M. Fuentes, L. Betancor, V. Grazu, F. López-Gallego, B.C.C. Pessela, A. Hidalgo, G. Fernández-Lorente, R. Fernández-Lafuente, J.M. Guisan, Enzyme Microb. Technol. 37 (2005) 456–462.
[12] F. López-Gallego, T. Montes, M. Fuentes, N. Alonso, V. Grazu, L. Betancor, J.M. Guisan, R. Fernández-Lafuente, J. Biotechnol. 116 (2005) 1–10.
[13] J.M. Palomo, R.L. Segura, G. Fernandez-Lorente, R. Fernandez-Lafuente, J.M. Guisan, Enzyme Microb. Technol. 40 (2007) 704–707.

[14] R.M. Blanco, P. Terreros, N. Muñoz, E. Serra, J. Mol. Catal. B: Enzym. 47 (2007) 13–20.
[15] K. Kawakami, S. Yoshida, J. Ferment. Bioeng. 82 (1996) 239–245.
[16] J.M. Palomo, G. Muñoz, G. Fernández-Lorente, C. Mateo, M. Fuentes, J.M. Guisán, R. Fernández-Lafuente, J. Mol. Catal. B: Enzym. 21 (2003) 201–210.
[17] M. Arroyo, J.M. Sánchez-Montero, J.V. Sinistera, Enzyme Microb. Technol. 24 (1999) 3–12.
[18] P. Lozano, T. De Diego, T. Sauer, M. Vaultier, S. Gmouh, J.L. Iborra, J. Supercrit. Fluids 40 (2007) 93–100.
[19] Z. Knezevic, N. Milosavic, D. Bezbradica, Z. Jakovljevic, R. Prodanovic, Biochem. Eng. J. 30 (2006) 269–278.
[20] M. Matsumoto, K. Ohashi, Biochem. Eng. J. 14 (2003) 75–77.
[21] J.M. Palomo, C. Mateo, G. Fernández-Lorente, L.F. Solares, M. Diaz, V.M. Sánchez, M. Bayod, V. Gotor, J.M. Guisan, R. Fernandez-Lafuente, Tetrahedron Asymmetry 14 (2003) 429–438.
[22] G. Fernandez-Lorente, J.M. Palomo, Z. Cabrera, R. Fernandez-Lafuente, J.M. Guisan, Biotechnol. Bioeng. 97 (2007) 242–250.
[23] M.L. Foresti, M.L. Ferreira, Enzyme Microb. Technol. 40 (2007) 769–777.
[24] A.L. Paiva, V.M. Balcão, F.X. Malcata, Enzyme Microb. Technol. 27 (2000) 187–204.
[25] U. Derewenda, A.M. Brzozowski, D.M. Lawson, Z.S. Derewenda, Biochemistry 31 (1992) 1532–1541.
[26] J.M. Palomo, G. Muñoz, G. Fernández-Lorente, C. Mateo, R. Fernández-Lafuente, J.M. Guisan, J. Mol. Catal. B: Enzym. 19/20 (2002) 279–286.
[27] J.M. Palomo, M. Fuentes, G. Fernández-Lorente, C. Mateo, J.M. Guisan, R. Fernández-Lafuente, Biomacromolecules 4 (2003) 1–6.
[28] B. Krajewska, Enzyme Microb. Technol. 35 (2004) 126–139.
[29] W.S. Adriano, E.H.C. Filho, J.A. Silva, L.R.B. Gonçalves, Biotechnol. Appl. Biochem. 41 (2005) 201–207.
[30] P.W. Tardioli, J. Pedroche, R.L.C. Giordano, R. Fernández-Lafuente, J.M. Guisan, Biotechnol. Prog. 19 (2003) 352–360.
[31] R. Fernandez-Lafuente, C.M. Rosell, V. Rodriguez, C. Santana, G. Soler, A. Bastida, J.M. Guisan, Enzyme Microb. Technol. 15 (1993) 546–550.
[32] L. Betancor, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, G. Dellamora-Ortiz, C. Mateo, R. Fernández-Lafuente, J.M. Guisan, Enzyme Microb. Technol. 39 (2006) 877–882.
[33] S. Rocchietti, A.S.V. Urrutia, M. Pregnolato, A. Tagliani, J.M. Guisan, R. Fernandez-Lafuente, M. Terreni, Enzyme Microb. Technol. 31 (2002) 88–93.
[34] A. Bastida, P. Sabuquillo, P. Armisen, R. Fernández-Lafuente, J. Huguet, J.M. Guisán, Biotechnol. Bioeng. 58 (1998) 486–493.
[35] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[36] M.M. Beppu, E.J. Arruda, R.S. Vieira, N.N. Santos, J. Membr. Sci. 240 (2004) 227–235.
[37] L. Sundberg, J. Porath, J. Chromatogr. 90 (1974) 87–98.
[38] N. Li, R.B. Bai, Ind. Eng. Chem. Res. 44 (2005) 6692–6700.
[39] A. Sadana, J.P. Henley, Ann. N.Y. Acad. Sci. 501 (1987) 73–79.
[40] C. Mateo, O. Abian, R. Fernandez–Lafuente, J.M. Guisan, Enzyme Microb. Technol. 26 (2000) 509–515.
[41] R.S. Vieira, M.N. Beppu, Colloids Surf. A: Physicochem. Eng. Aspects 279 (2006) 196–207.
[42] S. Hirano, K. Nagamura, M. Zhang, S.K. Kim, B.G. Chung, M. Yoshikawa, T. Midorikawa, Carbohydr. Polym. 38 (1999) 293–298.
[43] C. Mateo, J.M. Palomo, M. Fuentes, L. Betancor, V. Grazu, F. López-Gallego, B.C.C. Pessela, A. Hidalgo, G. Fernández-Lorente, R. Fernández-Lafuente, J.M. Guisán, Enzyme Microb. Technol. 39 (2006) 274–280.
[44] C. Mateo, R. Torres, G. Fernandez-Lorente, C. Ortiz, M. Fuentes, A. Hidalgo, F. Lopez-Gallego, O. Abian, J.M. Palomo, L. Betancor, B.C.C. Pessela, J.M. Guisan, R. Fernandez-Lafuente, Biomacromolecules 4 (2003) 772–777.
[45] A.I.S. Brígida, A.D.T. Pinheiro, A.L.O. Ferreira, G.A.S. Pinto, L.R.B. Gonçalves, Appl. Biochem. Biotechnol. 136 (2007) 67–80.
[46] R.M. Blanco, J.M. Guisan, Enzyme Microb. Technol. 11 (1989) 360–366.
[47] P.W. Tardioli, R. Fernandez-Lafuente, J.M. Guisan, R.L.C. Giordano, Biotechnol. Prog. 19 (2003) 565–574.
[48] D.S. Rodrigues, G.P. Cavalcante, A.L.O. Ferreira, L.R.B Gonçalves, Chem. Biochem. Eng. Q., in press.
[49] S.S. Naik, N.G. Karanth, Appl. Biochem. Biotechnol. 28 (1978) 569–580.