Immobilization of *Candida antarctica* Lipase Type B by Adsorption on Activated Carbon

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The immobilization of *Candida antarctica* lipase type B on activated carbon was studied. Adsorption conditions were investigated in order to improve enzyme activity and stability in organic media. Results showed that biocatalyst activity and recovered activity were improved with increasing concentrations of ammonium sulphate on the supernatant during enzyme adsorption. Hydrophobic interactions were the driving force of the immobilization process. Nevertheless, the specific and recovered activity of the immobilized enzyme is affected by pH of adsorption, and best results were obtained when lipase adsorption was conducted near the enzyme isoeletric point (pI 6.0). Operational stability of the immobilized enzyme was markedly improved when lipase loading was increased from 74.15 U g^{-1} to 112.34 U g^{-1}. After the sixth cycle of butyl butyrate synthesis, it retained around 10 % of the initial activity. Derivatives prepared in this work were tested and compared to a commercial derivative and results showed that they were a suitable biocatalyst to be used in the synthesis of flavours, such as butyl butyrate.

Key words:
Enzyme technology, adsorption, immobilized enzyme, lipase, activated carbon

Introduction

Lipases (E.C. 3.1.1.3) constitute a group of enzymes that catalyze the hydrolysis of lipids in biological systems.[1] In organic media, the enzymatic behaviour changes and the enzyme can be used for the synthesis of different lipids.[2] The versatility of these enzymes leads to several industrial applications in food and flavour making, pharmaceuticals, synthesis of carbohydrate ester, amines and amides, cosmetics, among others.[3]

The enzyme studied in this work, *Candida antarctica* lipase B (CALB), is a globular α/β type protein with dimensions of 3.0 · 4.0 · 5.0 nm, molecular mass of 33 kDa and isoelectric point of 6.0.[2] CALB is not as efficient as other lipases in hydrolyzing triglycerides; however, it is highly stereospecific towards both ester hydrolysis and synthesis, due probably to a limited space available in its hydrophobic pocket. It also plays an important role in the synthesis of glucolipids.[2,4,5]

The stereospecificity of CALB, both in hydrolysis and in organic synthesis, make them attractive for applications in biochemical and industrial fields.[2] However, the recovery and re-usability of soluble enzymes as catalysts are quite limited, what has led to the development of a wide variety of immobilization techniques. Immobilized enzymes offer some operational advantages over soluble enzymes, such as choice of batch or continuous processes, rapid termination of reactions, controlled product formation, ease of removal from the reaction mixture and adaptability to various engineering designs.[6]

According to some authors,[7] the evaluation of lipase as biocatalysts for organic chemistry can be carried out, at laboratory scale, by using soluble enzymes or enzyme aggregates. However, the industrial use of such biocatalysts requires a suitable protocol for lipase immobilization, since the binding of lipases on pre-existing supports should greatly improve the performance of industrial reactors. Numerous methods have been developed for enzyme immobilization, which can be grouped into four major categories: physical adsorption, covalent binding to activated polymers, entrapment and occlusion, and crosslinking, using multifunctional reagents to link enzyme molecules to each other.[8]

In this work, adsorption was selected to immobilize CALB, since it is the oldest and simplest method for enzyme immobilization; however, the interactions involved are complex, including charge-charge, van der Waal's and hydrophobic interactions, and hydrogen bond.[9,10] Those interactions are reversible, but, in practice, several investi-

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gations show that protein does not desorb from the carrier without using detergents.[10,11] The advantages of immobilization by adsorption are its low cost, extreme simplicity, mild immobilization conditions, and possible regeneration of the support.

Some authors have studied lipase immobilization by adsorption on different supports.[12,13,14] *Candida antarctica* lipase B (CALB) was adsorbed on agarose gels coated with a dense layer of polyethylenimine (PEI-agarose) under very different experimental conditions of pH (5.0 to 9.0) and temperature.[13] The authors observed that different conformations of CALB, obtained by using different adsorption conditions, could be fixed by intense interactions involving enzyme and support surfaces, rendering biocatalysts with different properties. For instance, CALB immobilized on PEI-agarose at pH 9.0 was 4-fold more enantioselective and 8-fold more active than CALB immobilized at pH 5.0. The immobilization of *Candida rugosa* lipase on polypropylene powder EP100 was investigated and the authors[12] observed that commercial lipases showed a classical Langmuir adsorption pattern. Fed-batch produced lipases, however, presented a BET adsorption multilayer equation.

According to the literature,[14] adsorption isotherms describe the support affinity for lipase, while its shape reflects the lipase distribution on the support. When experimental data may be described by the Langmuir model, it implies that lipase forms a monolayer on the support. Thermodynamically, it indicates an energetically homogeneous surface where all sites are identical. Nevertheless, protein adsorption in not restricted to a monolayer on the support and adsorption of secondary layers has been reported.[10,14] This can be observed as a kink in the adsorption isotherm after an apparent saturation of the matrix.[10]

In this article, we discuss the influence of immobilization conditions, especially ionic strength and pH, over the properties of the immobilized enzyme, such as thermal and operational stabilities.

Materials and methods

Materials

Novozyme 435 and *Candida antarctica* lipase type B (CALB) was kindly donated by Novozymes Latin America Ltd. (Brazil). CALB was used as received, 276.7 U per gram of protein in solution. Methyl butyrate and Bovine Serum Albumin were from Sigma-Aldrich Chemical Co (St. Louis, USA). Butyric acid and butanol were purchased from Merk (Rio de Janeiro, Brazil). Molecular sieve 4 A ($Na_2O[Al_2O_3(5.0SiO_2)]12H_2O$) was from W.R. Grace & Co. (Massachusetts, USA). The support used for enzyme immobilization was activated carbon, SRD/21/1 grade; Mesh 10*35 US, from Speakman Carbons LTD. Other chemicals were of analytical grade.

Methods

Preparation of immobilized enzyme. Enzyme immobilization was obtained by contacting 1.0 mL of enzyme solution (211.3 or 319.41 U), with 0.1 g of activated carbon and stirred for 2 h 30 min at room temperature (28 °C), using a rotary apparatus.[15] The enzyme solution was prepared by dissolving the crude extract in different buffer solutions depending on the desired pH. Samples were taken along the time course of adsorption and both protein concentration and hydrolytic activity on the bulk solution were measured. The adsorbed amount was calculated from the difference between enzyme hydrolytic activities in the supernatant before and after adsorption. After immobilization, the biocatalyst was separated by vacuum filtration and rinsed with phosphate buffer 0.1 mol L^{-1} and pH 7.0 to remove any weakly adsorbed enzyme. Alternatively, amounts of ammonium sulphate were dissolved in phosphate buffer and added to the support simultaneous to enzyme immobilization. A control experiment, without the addition of the support was carried out at the same pH and temperature conditions in order to evaluate enzyme stability during immobilization.

Protein estimation. The protein concentration was determined according to the procedure described by Bradford[16] using bovine serum albumin as standard protein.

Assay of hydrolytic activity: Methyl butyrate hydrolysis. The hydrolysis of methyl butyrate was used to follow the soluble and immobilized enzyme hydrolytic activities. Experiments were performed using an automatic titrator (pHstat) and 50 mmol L^{-1} NaOH as titrating agent.[17] The pH was set at 7.0 and the temperature was 28 °C. The reaction initiated with the addition of 0.1 mL of the soluble enzyme solution or 0.1 g of immobilized enzyme to 30 mL of a methyl butyrate (φ = 1 %) solution dissolved in phosphate buffer 25 mmol L^{-1} and pH 7.0. In this work, one unit (U) of enzymatic activity was defined as the amount of enzyme that hydrolyzes 1 μmol of methyl butyrate per minute at pH 7.0 and 28 °C.

Immobilization yield and recovered activity. The immobilization yield was defined as the difference between enzyme activity in the supernatant before and after immobilization divided by the en-

zyme activity in the supernatant before immobilization. Recovered activity was defined as the ratio of enzymatic activity of the immobilized enzyme and the total units of soluble lipase that disappeared from the supernatant during immobilization, measured by the hydrolysis of methyl butyrate at pH 7.0 and 28 °C.

Operational stability. The synthesis of butyl butyrate was used to monitor the biocatalyst esterification activity, based on a method presented in the literature.[18] Stock solutions of butyric acid (0.3 mol L^{-1}) and butanol (0.3 mol L^{-1}) were prepared in *n*-heptane. Experiments were set up in 15 mL flasks containing 5 mL of stock solution and 0.2 g of the biocatalyst (74.15 U g^{-1}, 112.34 U g^{-1} or 141.81 U g^{-1}). The flasks were kept at 28 °C under vigorous agitation for 3 h. The consumption of butyric acid was measured by titration with 0.02 mol L^{-1} NaOH and using phenolphthalein as indicator. The total acid content before reaction was determined by titration of a blank sample, without enzyme. Operational stability of the immobilized enzyme was assayed in successive batches of butyl butyrate synthesis. At the end of each batch, the immobilized lipase was removed from the reaction medium, washed with phosphate buffer to remove any remaining substrate or product, and hydrolytic activities were assayed as described above. Thereafter, the derivative was introduced into a fresh medium.

Assays of thermal stability. Soluble and immobilized enzymes were incubated in a phosphate buffer 0.1 mol L^{-1} and pH 6.0 at 50 °C. Periodically, samples were withdrawn and their residual activities were assayed by the hydrolysis of methyl butyrate. In this work, residual activity is given as a percentage of initial activity (before incubation). The deactivation coefficient and half-life ($t_{1/2}$) for each immobilized derivative was calculated according to eq. (1) and (2), respectively.[19]

$$\ln at = \ln at_0 - k_d t \quad (1)$$

$$t_{1/2} = \frac{\ln 0.5}{-k_d} \quad (2)$$

where at_0 is the initial hydrolytic activity and at is the hydrolytic activity after thermal treatment at 50 °C for a given time (t).

Amount of adsorbed protein. In order to obtain the amount of adsorbed protein to the solid phase, a mass balance was used, described by eq. (3).

$$q_s = \frac{V_f}{m_s}[\gamma_0 - \gamma(t)] \quad (3)$$

where q_s is the amount of adsorbed enzyme (U g^{-1}); V_f is the volume of liquid phase (mL); m_s is the mass of solid phase (g); γ_0 is protein mass concentration (mg mL^{-1}) or lipase activity (U mL^{-1}) in the liquid phase (supernatant) before adsorption, and $\gamma(t)$ is the residual lipase activity or protein mass concentration in the supernatant (U mL^{-1} or mg mL^{-1}) at any given time.

Adsorption isotherms. Adsorption equilibrium is represented by an isotherm, which is obtained by plotting enzyme loading (amount of adsorbed enzyme per gram of support) q (U g^{-1}) against residual lipase concentration in the solution after immobilization.[14,20,21] Several adsorption isotherms are related in the literature,[14] in this work, experimental data were fitted to Langmuir isotherm (eq. (4)) and model parameters were estimated using Software Origin version 6.0.

$$q = \frac{q_{max}\gamma_E}{\gamma_E + K_L} \quad (4)$$

where γ_E is lipase mass concentration in solution after immobilization (mg mL^{-1}); q_{max} is the maximum amount of adsorbed enzyme; q is the amount of adsorbed enzyme, and K_L is the Langmuir constant.

Determination of specific surface area of the support. Nitrogen isotherms were measured at 77 K with Autosorb-1 MP apparatus. Nitrogen isotherms were obtained in both adsorption and desorption modes. The surface area of the support and biocatalysts were determined by the BET (Brunauer, Emmett and Teller) method.[22]

Enzyme purification. CALB crude extract was submitted to dialysis, which was carried out using a cellulose acetate membrane.[23] It was dialyzed overnight at 4 °C against 5 mmol L^{-1} phosphate buffer, pH 6.0, using a 35 mm diameter dialysis membrane (Fundação Sardi, São Paulo, Brasil) with a molecular mass cut-off of 14 kDa.

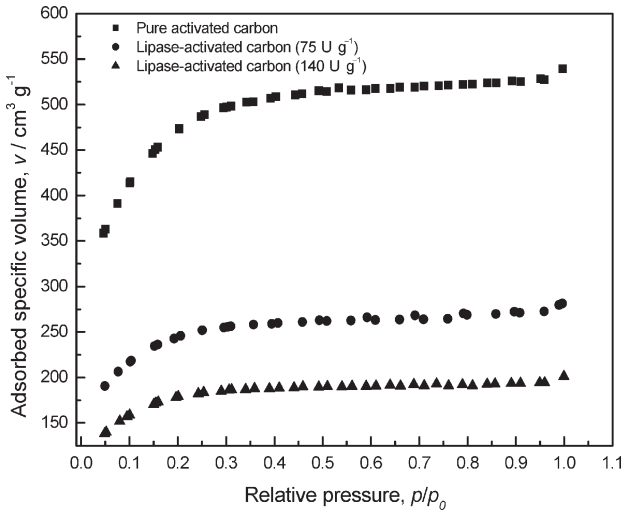
Electrophoresis assay. SDS-PAGE was carried out using 12 % polyacrylamide gels using an eletrophoresis unit (miniVE, Amersham Pharmacia Biotech). It was accomplished according to the method of Laemmli.[24] Protein bands were detected by Coomassie staining[25] and the silver nitrate method,[26] using BSA as standard.

Results and discussion

Surface and pore size distribution before and after immobilization

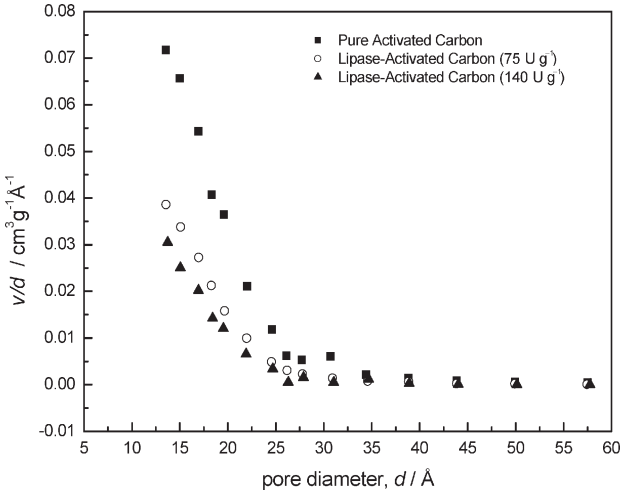
First, the support used for lipase immobilization was characterized in terms of physical properties, such as superficial area and mean pore diameter. According to other authors,[27] these properties could have an effect on the enzyme loading of the support.

The nitrogen adsorption-desorption isotherms, BET isotherms, of pure activated carbon (protein free) and lipase-activated carbon (immobilized enzyme) were obtained and the results are presented in Fig. 1. By analyzing its shape, it can be observed that they match type I isotherms, according to IUPAC, which corresponds to a microporous material, with pores not exceeding 2.0 nm.[28]



F i g . 1 – *Nitrogen adsorption isotherms of different samples of activated carbon*

Pore diameter data is presented in Fig. 2. Despite high surface area, little of it is available for enzyme immobilization, since the mean pore diameter, 1.35 nm, is smaller than the CALB molecule, 3.0 · 4.0 · 5.0 nm.[29] Large regions of the surface, determined based on the adsorption of N_2 molecules, are located in cavities into which the protein cannot penetrate and, thereby the binding area available to CALB is reduced.[10] Therefore, the enzyme must be immobilized on the superficial area of the support. Similar results were obtained by other authors[27] when studying the factors that affected esterification of lauric acid using commercially immobilized lipases, Lipozyme®. They demonstrated, by electron microscopy, that the enzyme did not penetrate into the internal surface of the particles when the mean pore diameter of the support was smaller than the enzyme molecule.



F i g . 2 – *Pore size distribution of pure activated carbon and lipase-activated carbon*

Table 1 shows the particle surface area, estimated from the BET isotherms. As it can be observed, the surface area decreases when enzyme is adsorbed on activated carbon, indicating enzyme immobilization on the support. It can be noticed that when enzyme load was doubled, from 74.14 U g^{-1} to 141.81 U g^{-1}, the superficial area reduced by 1.4-fold. This may be an indication that the distance between enzyme molecules in the support had diminished when lipase concentration was enhanced. In other words, when high enzyme load was used, the protein molecules were probably immobilized at close proximity to each other, which may have prevented deactivation caused by enzyme unfolding due to strong interaction with the support.

T a b l e 1 – *Experimental data obtained from adsorption isotherms of nitrogen on different samples of activated carbon*

Samples	Specific surface area a/m^2 g^{-1}
pure activated carbon	1491
lipase-activated carbon (74.15 U g^{-1})	777
lipase-activated carbon (141.81 U g^{-1})	548

No desorption of CALB occurred during the assay of hydrolytic activity, since no increase on surface area was observed after methyl butyrate hydrolysis and no protein was detected on the supernatant (data not shown).

Effect of ionic strength in the adsorption of *Candida antarctica* lipase B to activated carbon

According to some authors,[30] the rate and yield of hydrophobic adsorption of proteins increase when high concentrations of ammonium sulphate are present. They also observed that most proteins present in the crude extract were not adsorbed under very mild conditions (low ionic strength).

Table 2 shows the experimental results for the immobilization of *Candida antarctica* lipase B on activated carbon at pH 6.0, 28 °C in the presence of different concentrations of ammonium sulphate. It can be observed that both the activity of immobilized lipase and the recovered activity improved with increasing concentrations of ammonium sulphate, while the immobilization yield improved only when 1 mol L^{-1} ammonium sulphate was added to the medium. The support used in this work (activated carbon) has a high specific surface area (1400 m^2 g^{-1}) and an average pore diameter $\langle d \rangle$ = 1.35 nm. Compared with CALB dimensions (3.0 · 4.0 · 5.0 nm), it can be observed that the enzyme was immobilized on the external area of the support. At low ionic strength, only lipase molecules adsorbed on the surface, promoting a change in enzyme structure due to intense interactions between enzyme and support. When ionic strength is enhanced, other proteins, present in the crude enzymatic extract (Fig. 3), adsorbed to activated carbon and were capable of lipase stabilization, improving activity of immobilized CALB and recovered activity. In this case, those contaminant proteins act as additives that increase enzyme activity and stability. According to the literature, enzyme stabilization with an additive is achieved by preventing deactivation of the enzyme molecule during immobilization.[1] The contaminant proteins probably stabilize CALB by acting at the enzyme-support interface, preventing the enzyme from unfolding by covering the interface. Furthermore, non-covalent immobilization can be achieved by precipitating protein molecules from an aqueous solution. When enhancing the ammonium sulphate concentration, a common salt used for enzyme precipitation,[32] the precipitation of contaminant proteins on the support is favoured.

Table 2 – *Activity of immobilized lipase, obtained by adsorption at pH 6.0, 28 °C in the presence of different concentrations of ammonium sulphate. In all experiments, 319.41 units of soluble enzyme were initially offered to 0.1 g of support at 28 °C. Immobilization assays were performed in triplicate and standard deviations were estimated.*

Ammonium sulphate concentration c/mol L^{-1}	Immobilization yield γ/%	Immobilized enzyme activity a_E/U g^{-1}	Residual activity a_r/%
0.0	34.0	43.80 ± 4.5	5.65 ± 0.6
0.1	32.0	42.40 ± 0.3	8.75 ± 0.1
1.0	77.7	74.15 ± 4.5	13.4 ± 0.0

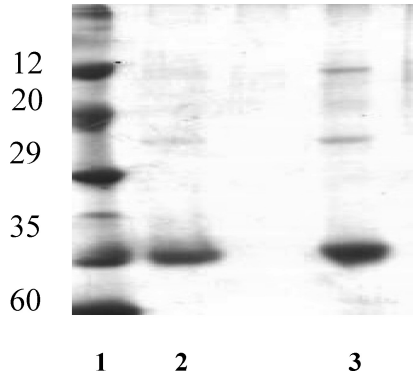


Fig. 3 – *Electrophoresis of CALB in 12 % polyacrylamide gels. Lane 1: molecular mass markers; 2: CALB pure; 3: crude extract*

Other authors[29,33,34] also described the loss of activity of CALB immobilized on hydrophobic supports. They attributed such inactivation to strong interactions with the support in derivatives with low lipase loading, causing a strong structural distortion. For instance, three commercial lipases, lipase A Amano 6 (from *Aspergillus niger*), lipase M Amano 10 (from *Mucor javanicus*), and lipase R Amano (from *Penicillium roqueforti*), were immobilized on Accurel MP1004 porous polypropylene by physical adsorption and the authors observed that the immobilization process caused a loss of enzymatic activity.[34] They observed that the retained activity was similar for lipase M and R (about 15 %). In contrast, lipase A retained only 1.3 % of the specific activity of the soluble lipase.

Fig. 4 shows the thermal inactivation of *Candida antarctica* lipase B adsorbed on activated carbon at pH 6.0, 28 °C in the presence of 1 mol L^{-1} of ammonium sulphate, here named lipase ADS, compared to the soluble enzyme. It can be observed that lipase ADS is more stable than the corresponding soluble enzyme at pH 6.0 and 50 °C. From the data presented in Fig. 4, thermal deactivation constants and half-lives of soluble and immobilized enzyme were obtained. The deactivation coefficient (K_d) was 0.14 h^{-1} and 0.09 h^{-1} to the soluble and immobilized enzyme, respectively. The half-life of the soluble enzyme at 50 °C was 4.8 h, whereas the half-life of the immobilized enzyme increased to 8.0 h. These results indicate that immobilization preserves the enzyme structure from inactivation.

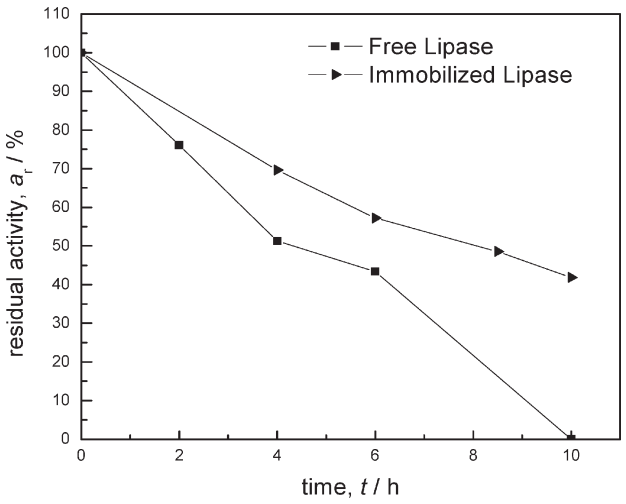


Fig. 4 – *Thermal inactivation of Candida antarctica lipase B adsorbed to activated carbon at pH 6.0, 28 °C in the presence of 1 mol L^{-1} of ammonium sulphate (lipase ADS) compared to the soluble enzyme. Experiments were performed at pH 6.0 and 50 °C, and residual activity was monitored by methyl butyrate assay. Squares: Soluble enzyme. Triangles: lipase ADS. (74.15 ± 4.5 U g^{-1}).*

Effect of pH in the adsorption of *Candida antarctica* lipase B to activated carbon

The hydrophobic nature of the activated carbon used as support implies that the enzyme adsorption is governed by hydrophobic interactions. Therefore, those interactions should not be affected by changes in the pH of adsorption. On the other hand, if electrostatic forces are important, changes over the isoelectric point of lipase will have a large impact on the binding constants.[10]

The kinetic results of protein adsorption on activated carbon are presented in Fig. 5. The pH values chosen correspond to positive (pH 5.0), electroneutral (pH 6.0), and negative (pH 7.0 and pH 8). In all cases, two parts on the kinetic curve can be observed: an initial linear part and a plateau region. Adsorption is faster at the initial linear stage and the amount of protein is proportional to time. The time to achieve equilibrium between adsorbed and solution protein molecules were dependent on pH, and it remained around 60 min at pH 6.0 and 120 min at pH 5.0, 7.0 and 8.0.

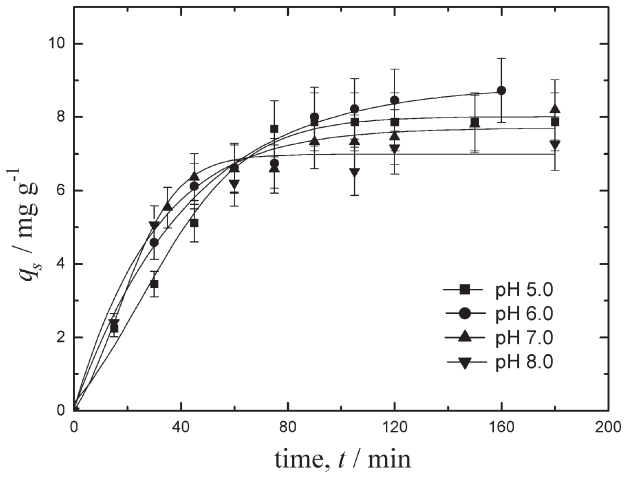


Fig. 5 – *Kinetics of protein adsorption on activated carbon at different pH's. Immobilization conditions: 28 °C and 8.16 gram of protein per gram of support*

By analyzing the data shown in Fig. 5, no important differences in the amount of bound protein were observed for the different values of pH studied, supporting the hypothesis of hydrophobic interactions being the driving force of the immobilization process. Nevertheless, the immobilized CALB activity and recovered activity is affected by the pH of adsorption, as can be observed in Table 3. Best results were obtained when adsorption was conducted near the enzyme isoeletric point (pI 6.0), suggesting that, at this pH, the enzyme is immobilized in its active configuration. Similar results were obtained by other authors[30,35] when immobilizing a *Candida rugosa* lipase on poly(acrylonitrile-co-maleic acid) hollow fibre and *Mucor javanicus* lipase on SBA-15 mesoporous silica. According to them, lipase immobilization in pH range of 5.0 – 6.5 provided relatively high activity values because enzyme conformation, vital for enzymatic activity, changed with pH. The ionization state of the active site of the lipase molecule is affected by the pH of the buffer used in the immobilization process and activity is very sensitive to the pH of the solution during the binding step.[36]

Table 3 – *Effect of pH of adsorption on immobilized CALB activity and recovered activity. In all experiments, 211.3 units of soluble enzyme were initially offered to 0.1 g of support at 28 °C.*

pH	Immobilized enzyme activity a_E/U g^{-1}	Residual activity a_r/%
4	78.60 ± 1.1	6.84 ± 0.5
5	88.75 ± 0.2	7.15 ± 0.5
6	83.30 ± 4.5	6.58 ± 0.2
7	56.45 ± 2.8	4.29 ± 0.6
8	62.50 ± 0.4	5.07 ± 0.0
9	68.75 ± 0.9	8.91 ± 2.7
10	46.80 ± 8.2	5.85 ± 0.8

Adsorption isotherms at different pH values

Adsorption isotherms describe the support affinity (or capacity) for lipase, while the isotherm shape reflects lipase distribution on the support surface.[14] Protein adsorption is not restricted to a monolayer cover on the carrier. Adsorption of secondary protein layer has been reported and can be observed as a kink in the adsorption isotherm after an apparent saturation of the carrier.[9]

Enzymatic loading is usually expressed in milligrams of immobilized enzyme per gram of support. Since different proteins (contaminant proteins) may be immobilized during the process, enzyme loading was expressed in terms of activity per gram of support; in order to assure it is proportional to the amount of active lipase effectively immobilized.[35] Fig. 6 shows CALB adsorption to activated carbon at two different pH values. No kink in the isotherms was observed in the range of concentration studied. Furthermore, both equilibrium curves were described by the Langmuir model, which implied the formation of a monolayer on the support surface. Thermodynamically, this indicates an energetically homogeneous surface where all sites are identical.[14] There were no significant differences in the binding constants for the pH values studied (Table 4), supporting the theory that hydrophobic interactions were the driving force of lipase adsorption.

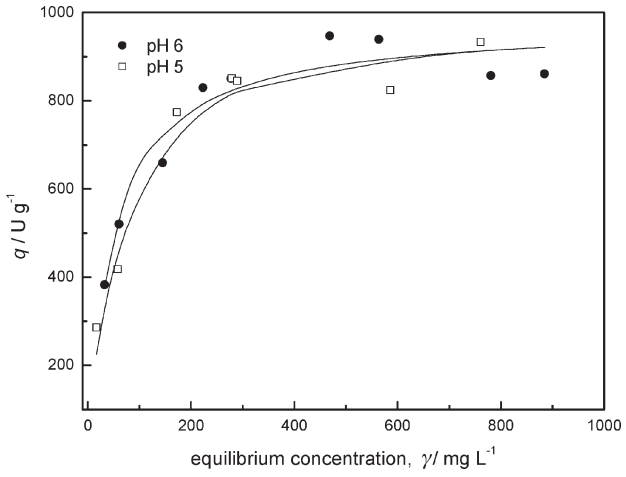


Fig. 6 – *Adsorption isotherms for CALB on activated carbon, estimated at different pH and room temperature*

Table 4 – *Values of Langmuir constants (K_L) and maximum lipase loading (q_{max}) for CALB immobilized on activated carbon at different pH values*

pH	K_L/U mL^{-1}	q_{max}/U g^{-1}
5	55.58 ± 13.8	979.32 ± 50.8
6	51.24 ± 9.4	974.45 ± 34.4

Synthesis of butyl butyrate using different biocatalysts

Synthesis of butyl butyrate was used to evaluate esterification activity of lipase ADS and Novozymes 435, a commercial biocatalyst. The consumption of butyric acid, after 24 h of reaction, was measured by titration and compared to the total acid content before reaction. Best results of synthetic yield, defined as butyric acid consumption, were obtained with Novozymes 435, 92.1 % molar yield. However, a higher synthetic yield was obtained with lipase ADS (84.8 % molar yield), what shows that it is a suitable biocatalyst for the synthesis of flavours, such as butyl butyrate.

Operational stability studies

Operational stability of immobilized enzymes is very important economically and an increased stability could make the immobilized enzyme more advantageous than its soluble counterparts.[37] Therefore, in this work, the effect of repeated use on lipase activity was investigated in the synthesis of butyl butyrate in heptane (Fig. 7). It can be observed that lipase ADS with 74.15 U g^{-1} showed poor operational stability, loosing more than 80 % of its initial activity after the first cycle of reaction. However, operational stability of the immobilized enzyme was markedly improved when lipase loading increased from 74.15 U g^{-1} to 112.34 U g^{-1}. No significant improvement on operational stability was obtained increasing enzyme load from 112.34 U g^{-1} to 141.81 U g^{-1}.

It can be observed that when high loads of enzyme were immobilized on activated carbon (112.34 or 141.81 U g^{-1}), the biocatalyst could be

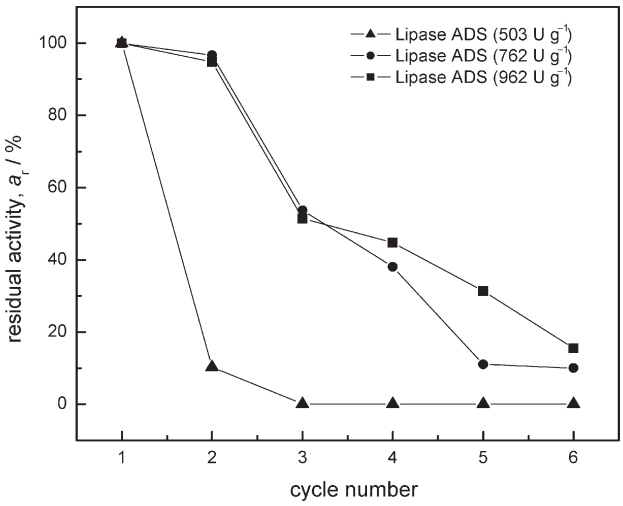


Fig. 7 – *Molar yield of butyric acid consumption during butyl butyrate synthesis in subsequent reaction cycles catalyzed by different amounts of CALB adsorbed on activated carbon (Lipase ADS)*

used up to three cycles with less than 50 % loss of activity. After six cycles, however, the immobilized enzyme retained only 10 % or 15 %, respectively, of its original activity. Based on these results, we can conclude that the enzyme immobilized on activated carbon show a high initial activity but it decreases during successive uses. The same behaviour was observed by other authors[21,38] when studying the immobilization of *Candida cylindracea* and *Candida rugosa* lipase by adsorption. When *Candida cylindracea* was immobilized on zeolite type Y, the immobilized enzyme retained, after 7 cycles, only 10 % of its initial stability in the hydrolysis of palm oil.[21] Both niobium oxide (crystalline and amorphous) supports, used for the immobilization of *Candida rugosa*, showed poor operational stability resulting in high activity loss (over 75 %) after five recycles.[38]

The physical adsorption method is relatively easy and simple for immobilization and leads to minimal structural change of the enzyme during the immobilization process. However, bonding forces such as hydrogen bonds, Van der Walls forces, and/or hydrophobic interactions are not strong and do not protect the enzyme from conformational changes (unfolding), that may be caused by the interaction with the solvent during the esterification reaction. To overcome this, it is recommended to use a high load of enzyme so that a suitable amount of protein can be spread on the surface area, avoiding conformational changes.[38] Nevertheless, it is important to have in mind that, at lower loading, the specific activity is reduced by unfolding of the enzyme on the support. In contrast, at higher loading, unfolding of the native enzyme is less severe, but diffusional limitations may suppress activity.[39,40]

Conclusions

According to experimental work, activated carbon is a suitable support for lipase immobilization. Best results were obtained when lipase was adsorbed on activated carbon in the presence of 1 mol L^{-1} of ammonium sulphate, probably because other proteins present in the enzyme crude extract adsorbed to activated carbon and were capable of lipase stabilization, improving the activity of the derivative and recovered activity. Although the amount of *Candida antarctica* lipase type B adsorbed to activated carbon was independent of the pH of adsorption, indicating that hydrophobic interactions were the driving force of the immobilization process, immobilized enzyme activity and recovered activity were dependent on the pH of adsorption. Best results were obtained when lipase adsorption was conducted near the enzyme isoeletric point (pI 6.0), due to a favourable charge distribution on the amino acid residues, which prevented a possible change of enzyme conformation. At 50 °C, the adsorbed enzyme was 2-fold more stable than the soluble enzyme. Finally yet importantly, the molar yield of butyric acid consumption, during the synthesis of butyl butyrate, compared to a commercial derivative, showed Lipase ADS is a suitable biocatalyst to be used in the synthesis of flavours.

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List of symbols

a – specific surface area, $m^2\ g^{-1}$
a_E – enzyme activity, U g^{-1}, g L^{-1} h^{-1}
a_h – hydrolytic activity, U mL^{-1}
a_r – residual activity, %
c – concentration, mmol L^{-1}, mol L^{-1}
d – pore diameter, Å, nm
K – equilibrium Langmuir constant, mg L^{-1}
K_d – deactivation coefficient, h^{-1}
m – mass, mg, g
p – pressure, Pa
p/p_0 – relative pressure
pI – isoelectric point
q – lipase loading, U g^{-1}
q_s – adsorbed capacity, mg g^{-1}
t – time, h
v – adsorbed volume, $cm^3\ g^{-1}$
γ – mass concentration, g L^{-1}
φ – volume fraction, %

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