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Catalysis by *Candida antarctica* B (CALB) immobilized on natural pure silica by adsorption: comparison with the free enzyme

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ABSTRACT

Objective: In this work, a lipase B from *Candida antarctica* strain was immobilized onto natural silica carriers via adsorption to enhance its feasibility in practical applications.

Methodology and results: The biocatalyst was prepared by simple adsorption on the support whose composition was beforehand characterized and the activities in ethyl ester hydrolysis and synthesis evaluated. The natural silica carriers were rich in silicon dioxide (SiO_2) (97.36%). The optimum temperature of immobilized lipase was 40 °C, which was identical to that of the free enzyme. The immobilized lipase exhibited a higher relative activity than that of free lipase over 40 °C. The optimal pH for immobilized lipase was 7.5, which was similar to that of the free enzyme. After 10 recycled batches, a high residual esterification conversion (90%) was maintained.

Conclusions and application of findings: The results in the present work indicate that pure silica is a potential material as an immobilization matrix for industrial process. There may have a promising future as support of the biocatalysts in various syntheses.

Key words: Lipase immobilization, adsorption, *Candida Antarctica*, natural silica.

INTRODUCTION

Lipase (triacylglycerol ester hydrolases, EC 3.1.1.3) is an important enzyme with a broad variety of industrial applications due to the multiplicity of reactions, which it catalyzes, with a broad variety of applications in fine chemistry, pharmaceutical industry and in the food industry owing to their usefulness in both hydrolytic and synthetic reactions (Sharma *et al*., 2001; Monier *et al*., 2010). Several works were carried out these last years to obtain immobilized lipase derivatives suitable for different reactions. An immobilized enzyme is defined as the enzyme physically confined to a certain defined

region while retaining its most catalytic activity (Jegannathan *et al*., 2008). Among the several methods of immobilization, adsorption is the method, which induces less modification on the active conformation of the enzymes. Consequently, adsorption is one of the methods preferred for the immobilization of enzymes. In this method, enzyme fixation is performed through hydrogen bonds, salt linkages, and Van der Waal's Forces. The use of phyllosilicates, mainly sepiolite, to immobilize enzymes was described; nevertheless, silicate-type materials have been usually used in a two-step covalent immobilization method, which involves the previous activation of the clay and a posterior covalent binding of the enzyme. The use of silicates as lipase carriers presents numerous advantages, such as the high specific surface available between (200 and 800 m2/g), the facility of water dispersion recuperation, the high water uptake capacity and the excellent mechanical resistance of these materials. Their natural origin and low cost make them even more attractive from an applied point of view. Efficiency and recyclability of immobilized enzymes depend not only on the procedure and support utilized but also on the specific enzyme used and the type of process where it is applied (Li *et al*., 2013). Various immobilization procedures like adsorption, cross-linking, encapsulation or entrapment have been employed on lipases used for biodiesel production (Jegannathan *et al*., 2008). However, most immobilization procedures use sophisticated protocols for lipase entrapment on expensive supports (Ferrer *et al*., 2002) not suitable for a real scale-up, causing an increase in the costs of industrial processes. Therefore, adsorption, used in this work, is a suitable system for lipase immobilization that could be successfully applied to large volume industrial processes, as in biodiesel production, because of its simplicity and low cost (Cesarini *et al*., 2014). This type of immobilization occurs *via* binding of the lipase onto the surface of the support by weak forces, such as Van der Waals or hydrophobic interactions or through dispersion forces.In this study, the physical adsorption of *Candida antarctica* B onto natural support rich in silica is described. The stability and the activity of the immobilized lipase were also investigated. Finally, the reusability of this immobilized lipase was tested.

MATERIALS AND METHODS

Chemicals: Lipase powder from *Candida antarctica* B was used as enzymes. Oleic acid and *Jatropha curcas* oil were used as substrate triglyceride. Ethanol was used for reaction. The oleic acid was bought and *Jatropha curcas* oils were obtained by the method of Soxhlet at the laboratory. Other chemicals and solvents were obtained from Chemical Company (Sigma Aldrich).

Characterization of support: Silica was crushed by a vibro-crusher (SIMENS SIMATIC BREADS 2) for 30 second. The loss on the ignition of the samples were determined by calcination with the furnace with 975C (± 25) during one hour to return the samples dryness before the analysis. The density was determined by the densimeter of Chatelier. The chemical composition of natural support rich in silica is determined with a spectrophotometer with ray-X (THERMO SCIENTIFIC, ARL OPTIM' X, WDXRF Spectrometer)

Immobilization of lipase: For immobilization in aqueous media 150 mg lipase was dissolved in 5 ml of sodium phosphates buffer (0, 1 mol /L, pH 7,3) and centrifuged at 2400× g for 3 min to remove insoluble components. The supernatant (4 ml) was mixed with pure silica (250 mg) under low stirring for 12h at room temperature. The particles were filtered under reduced pressure and washed with the same buffer under reduced pressure, and then one dried at room temperature. Consequently, the residue was obtained which is immobilized lipase on support. The immobilization efficiency was evaluated in terms of protein yields by measuring the difference between the protein concentration in the lipase solution before and after immobilization according to the following equation:

Protein immobilization yield (%) = (Ci-Cf)/Ci

where Ci is the initial protein concentration in the lipase solution, and Cf is the final protein concentration in solution after immobilization. The protein concentration was measured according to the method using bovine serum albumin (BSA) as the standard (Wrolstad 2000).

Hydrolytic activity: The hydrolytic activities of free and immobilized lipase were assayed by the palm oil emulsion method, according to the modification proposed by Soares *et al*. (Soares *et al*., 1999). The reactions of

hydrolysis of free and immobilized lipases were performed in screw-capped flasks containing 1g oil diluted in 5 ml of Cyclohexane to which one adds 20 µL of a calcium chloride solution (0,2M) and 50 µL of the enzymatic solution (3mg/L) prepared in sodium phosphates buffer (pH = 7). The mixture thus obtained was mixed under stirring (300 rpm) for 30mn at room temperature. The reaction stops when one adds 3.5 ml of an ethanol/acetone mixture. Free acidity is titrated (V) with a solution welds (C) in the presence of phenolphthalein against a white (Vo) without enzyme. The enzymatic activity (AE) is expressed in mole of free fatty acids which releases one mg of enzyme for unit of time (T; mn) according to the following formula: $AE = (C (V - Vo)) / (m \times t)$, with m mass of enzyme in the enzymatic solution.

Esterification assay: The esterification reactions were performed in screw-capped flasks with a molar ratio of oleic acid to ethanol 1:1 (0.4 mmol of oleic acid and 0.8 mmol of ethanol), of immobilized lipase dissolved in 4 ml of anhydrous n-hexane. The reaction mixture was shaken for 8 h at 220 rpm at 37°C in a shaking incubator. Aliquots of 200 µl were withdrawn periodically from the reaction mixture. The immobilized enzyme was removed by centrifugation at 2000 rpm for 5 min, and then the supernatant residual acids contents were assayed by titration with 0.5 N sodium hydroxide, using phenolphthalein as an indicator and 2 ml of ethanol as a quenching agent. The conversion (%) in ester synthesis was based on acid consumed (Bovora *et al*. 1993).

Enzymatic synthesis of biodiesel: The enzymatic transesterification reactions were carried out in a 50-mL shaking flask under magnetic stirring at 180 rpm at 40 ºC for 48 h, using a ratio (*Jatropha curcas* oil: ethanol) = (1:10) ; free or immobilized lipase = 5% of Jatropha curcas oil. The ethyl ester contents in the reaction mixture were quantified using a GC-2010 gas chromatograph.

Effect of temperature on the free and the immobilized lipase activities: The effect of temperature on the relative activity of the free and immobilized CALB was determined at pH 8.5 in temperature range varying from 25 to 50 C, oleic acid oil emulsion as substrate. Relative activities were calculated as the ratio of the enzyme activity measured at different temperatures to the maximal activity of the enzyme measured as described above.

Effect of pH on the free and the immobilized lipases activities: The effect of pH on the free and immobilized lipase activities was studied in pH range varying from 7.5 to 11 at 37◦C using olive oil emulsion as substrate by using different buffers: glycine–HCl 50 mM (pH 3–4), sodium acetate 50 mM (pH 5–6), phosphate 50 mM (pH 7), Tris–HCl 50 mM (pH 8–9) and boric acid 50 mM (pH 10–12). Relative activities were calculated as the ratio of the activity of enzyme measured at different pH to the maximal activity of enzyme, at pH 8.5 and 37◦C.

Reusability of the immobilized *Candida antartica* B lipase: The esterification of oleic acid with ethanol was conducted under these conditions (ethanol /oleic acid molar ratio equal to 1; enzyme; hexane volume of 3 ml and reaction time of 8 h) using lipase immobilized. This immobilized CALB was reused many times for consecutive cycles. At the end of each batch, the immobilized lipase was removed from the reaction medium, washed with n-hexane in order to remove any substrate or product retained in the support and dried at room temperature. Then, the immobilized lipase was used again for another reaction cycle using fresh substrates.

Statistical analysis: Experimental results were given as mean value ±SD of three parallel measurements. All statistical analysis was conducted using Microsoft Excel and software SPSS version 14.0 for the analysis of the variance was used for the comparison of the average.

RESULTS AND DISCUSSION

Activated carbons are among the most effective adsorbents, having high surface area per unit mass (Yesiloglu 2005). Due to the relatively high cost of activated carbons, there have been attempts to utilize low cost, naturally occurring adsorbents for immobilization. In recent years, there has been increasing interest in utilizing natural clay minerals like montmorillonite (Ramos *et al*., 2014), bentonite (Yesiloglu 2005), and sepiolite (Myriam *et al*.,1998) or natural polymers (Lv *et al*., 2008, Ittrat *et al*.,2014, Pereira *et al*.,2003, Ramani *et al*.,2010) can also be used as support for the immobilization of enzyme. They are natural matrices resulting from agricultural waste, which contain cellulose, hemicellulose and lignin for this process. Silica exists in the natural form in the world. In this work, it was used as support of immobilization considering its adsorbing character and its availability.

Chemical composition of pure silica and activity: The chemical composition of pure silica, as determined by spectrophotometer ray-X, was SiO2 (97.36%), aluminium oxide (Al_2O_3) (1.12%), magnesium oxide (MgO) (0.01%), (calcium oxide) CaO (0.22%), iron oxide (Fe2O3) (0.53%), sodium oxide (Na_2O_3) (0.03%), titanium oxide (TiO2). The silica content comparable with that is

contained in bentonite support (70 - 80 %) (Yesiloglu 2005), and largely higher than is contained of montmorillonite (50-60 %) (Ramos *et al*., 2014), sepiolite (69.1%) and the palygorskite (70%) (Myriam *et al*., 1998). Table 1 presents the results of immobilization and the free and immobilized lipase acitivity. The enzyme immobilized on pure silica showed the same protein immobilization yield of 42.63% approximately. The hydrolytic activity values were 5.94 µmol / (mg min and 2.33-µmol /mg min respectively for free and immobilization enzymes while the esterification activity were 91.70 % and 41.54% of oleic conversion.

Tableau 1: Immobilization yield and activity

	mg of added protein/g of support	Loading (mg of added protein fixed /g of support)	% of immobilization	Hydrolytic activity (µmol /(mg min))	Esterification activity* (%)	Trans-esterification activity** (%)
CALB free	-	-	-	5.94±0.1a	91.70±1.2a	90.40±1.2a
CALB immobilized	42.63±1.1	22.41±0.5	52.56±1.5	2.33±0.05b	41.54±0.8b	44.08±0.8b

Biochemical properties of free and immobilized lipase
pH stability and optimum pH: The effect of pH on the stability of both free and immobilized lipase was determined by measuring the residual conversion of oleic acid in the pH range of 6.0–8.0 and the results were presented in figure 1. The pH profiles of the immobilized lipases were broader than that of the free enzyme. Furthermore, the optimum pH of the immobilized lipase was at pH 7.0–8.0, while the optimum pH of free lipase was at 7.0. The retained activity of the immobilized enzyme was improved both at lower and higher pH in comparison to the free enzyme. The results show that the immobilization methods preserved the enzyme activity over a wider pH range.

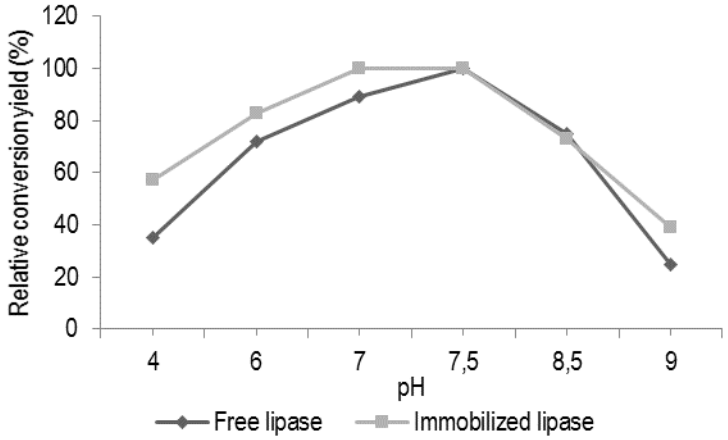

Figure 1: Effect of pH on lipase activity

Thermal stability: The immobilization of lipases onto solid support contributes to increase their thermostability and to extend their biotechnology potential, since running bioprocesses at elevated temperature is advantageous (Hasan *et al*., 2006). Both of the thermal stability of the free and the immobilized lipase was determined by measuring the residual conversion of oleic acid at 37°C after the enzyme exposed to temperatures ranging from 37 to 60°C in phosphate buffer (0.1 M, pH 7.5) for 30 min (figure 2). As observed in Figure. 2, the free and the immobilized enzyme exhibited different temperature profiles. The immobilized lipase was stable at 30°C, while the activity of the free enzyme was decreased drastically. The results also showed that the immobilized enzyme had more than 70% activity at 40°C, while the free enzyme had only 30% activity. Thus, the immobilized lipase was much more stable than the free enzymes at higher temperatures.

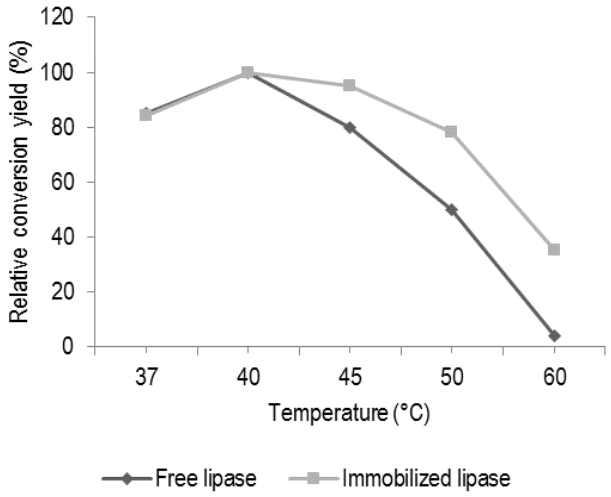

Figure 2: Effect of temperature on lipase activity

Reusability of the immobilized *C antartica* lipase: The main advantage of immobilization of an enzyme is that an expensive enzyme can be repeatedly used (Iso *et al*., 2001).Immobilized lipase was used repeatedly by measuring the residual conversion of oleic acid at 37 °C for 12h and the reusability was examined because of its importance for repeated applications in a batch or a continuous reactor. As shown in figure 3, lipase immobilized displayed a good reusability. The analysis of this figure shows a significant reduction in approximately 15% with the second use. This contact was also observed by several authors (Iso *et al*., 2001). When an immobilized enzyme was used for the first time, some amount of enzymes was desorbed. The desorption of an enzyme could not be observed after further repeated use. After 10 recycled batches, a high residual esterification conversion (70%) was maintained. This confirms the remarks of Bai *et al*., (2006) which show that the lipase immobilized on the supports mesoporous of silica (SiO_2) exhibit good thermal stability and reusability.

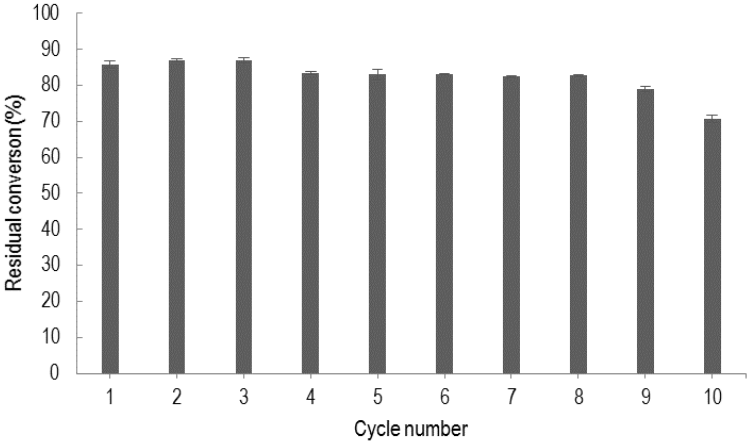

Figure 3: Effect of repeated use of immobilized lipase on the conversion of oleic acid

CONCLUSION

The results of the present study indicate that natural pure silica might be used as a support for *Candida antartctica* B. Immobilization of lipase can be carried out simply by direct deposition of the enzyme solution onto pure silica suspended in solvent free medium. Although enzymatic activity retained in the immobilized lipase (42%) was not impressive but it was comparable to many inorganic support materials. Results obtained in this work can

contribute to the development of immobilization processes of lipases using low-cost supports. Proper treatment of this support would undoubtedly improve its quality for use as supporting material for lipase immobilization. Thus, we recommend that more investigation, in the characterization of the adsorbent supports naturalness, is to realize in order to identify those, which can be used as supports of biocatalysts in several fields.

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