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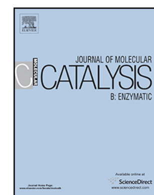

Immobilization of *Candida rugosa* lipase on bentonite modified with benzyltriethylammonium chloride

Margarita Díaz Ramos [a], Gloria Inés Giraldo Gómez [a,*], Nancy Sanabria González [b]

[a] Departamento de Física y Química, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Colombia – Sede Manizales, Cra 27 No. 64-60, Campus Palogrande, Manizales, Colombia
[b] Departamento de Química, Facultad de Ciencias, Universidad Nacional de Colombia – Sede Bogotá, Cra 30 No. 45-03, Bogotá, Colombia

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ABSTRACT

Two organobentonites with different hydrophobicities were used as supports for the immobilization of *Candida rugosa* lipase (CRL). The activities of the free and immobilized lipases were evaluated for the hydrolysis of olive oil. The organobentonites were prepared from sodium bentonite by interchange with benzyltriethylammonium chloride, using 1.0 and 2.1 times the cation exchange capacity (CEC) of the clay. The enzymatic activities of the lipase immobilized in the two organobentonites were 511.5 and 177.8 U/g support, while the activity recoveries were 33.5% and 14.7%. The operational stability of the immobilized enzyme as a function of pH and temperature was analyzed, and the resulting data were processed using analysis of variance (ANOVA) and response surface methodology (RSM). The conditions where the highest retention of activity for the CRL immobilized in the organobentonite using 1.0 times the CEC was found to be at a pH of 6.5 and a temperature of 39.6 °C. For the CRL immobilized in the organobentonite with 2.1 times the CEC, an ideal temperature was not found in the range evaluated and the optimal pH was determined to be 6.5. In general, the immobilization process preserved the enzyme structure in the interval of pH (5.5–7.0) and temperature (35–45 °C) studied and opens a broad range of possible industrial applications.

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1. Introduction

Lipases (triacylglycerol hydrolases; E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis and synthesis of lipids with great rapidity and excellent chemo-, regio- and enantio-specificities while operating under mild conditions [1]. Due to these characteristics of lipases, they have been applied in the oleochemical industry [2], detergent industry [3], resolution of racemic mixtures [4], production of additives for improving the taste and quality of food [5], cosmetics industry [6], production of biodiesel [7], and wastewater treatment [8], among others.

However, the industrial use of enzymes is affected by drawbacks, such as expensive isolation and purification processes, and once enzymes are isolated from their natural environment, their structure tends to be unstable. Furthermore, as enzymes are soluble, their recovery from the reaction mixture for reuse is very expensive [9,10]. The immobilization of enzymes is an option for their recovery and reuse as well as for maximizing their residual activity, which improves properties such as the operational stability, thermal stability, activity and selectivity [11,12].

The characteristics of immobilized enzymes depend on the enzyme structure, the immobilization method, and the type of support [11,13–15]. Numerous supports for the immobilization of lipases have been used and impart different catalytic properties to the enzyme [10,16,17]. The methods used include adsorption, ionic bonding, covalent binding, cross-linking, entrapment, and encapsulation. Adsorption is the simplest method and involves reversible surface interactions between the enzyme and support material. The forces involved in the adsorption process are typically hydrophobic interactions [18].

There are few studies available concerning the use of bentonite as a support for enzyme immobilization, although it has several advantages for use as a support, including its low cost, lack of toxicity, high surface area, and ability to switch from hydrophilic to hydrophobic character, thus allowing for the easy fixation of enzymes. Immobilized lipases may even increase their activity above the activity of the corresponding free lipase [12,19].

Previous experiments conducted with *Candida rugosa* lipase (CRL) non-covalently immobilized on a bentonite support showed higher thermal stability, and the pH shifted to a higher value when compared to the free enzyme. Additionally, the immobilized enzyme retained 30% of its activity [20]. Bentonite modified with cetyltrimethylammonium bromide (CTMAB) was used to support the lipase from a porcine pancreas. The immobilized lipase showed

* Corresponding author. Tel.: +57 6 8879300x50181; fax: +57 6 8879300x50342.
E-mail address: gigiraldogo@unal.edu.co (G.I. Giraldo Gómez).

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higher activity in the hydrolysis of olive oil and better resistance to pH and temperature inactivation compared to the free lipase. Additionally, after storage for 8 days, the immobilized lipase retained 80.7% of its initial activity, which was 2.7 times higher than the residual activity of the free lipase [21].

In the present work, two organobentonites with different hydrophobicities were prepared via cation exchange with various amounts of benzyltriethylammonium chloride (BTEAC) in sodium bentonite (Na-Bent) from Valle del Cauca-Colombia. This clay is the most commercially exploited in Colombia, a fact contributing to the valuation of a natural resource. The organobentonites were then employed as support materials for the immobilization of CRL through an adsorption process utilizing the hydrophobic interactions between the surfaces of the support and the lid of the lipases [12,22]. The changes in the structures of the bentonites before and after modification were investigated using analytical techniques, including X-ray powder diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR) and N_2 adsorption–desorption. To determine the catalytic activity of the free and immobilized CRL, the hydrolysis of olive oil was evaluated, which has been widely used as a model substrate in the lipase-catalyzed hydrolytic reaction. The operational stability of the immobilized lipase was studied using a range of pH and temperature conditions to find the optimal operating conditions that retain the highest hydrolysis activity. The obtained data were processed using analysis of variance (ANOVA) and response surface methodology (RSM).

2. Experimental

2.1. Reagents

C. rugosa lipase (≥700 units/mg solid) and benzyltriethylammonium chloride (BTEAC) (99%) were purchased from Sigma-Aldrich (St. Louis, MO, US). Bovine serum albumin (BSA) was purchased from Fluka – Sigma-Aldrich (St. Louis, MO, US) (≥96%). All other chemical compounds used in this study were of analytical grade. Deionized water (Millipore) was used to prepare all aqueous solutions.

2.2. Synthesis and characterization of organoclay

The starting clay was a natural bentonite from Valle del Cauca, Colombia, which was segregated by particle size (fraction <2 μm) following a gravimetric sedimentation procedure [23]. This bentonite is commercially exploited and sold in Panama, Ecuador, Venezuela and Central America. A fraction of the clay was homoionized with 1.0 M NaCl, washed repeatedly with deionized water until free of chlorides, dried at 60 °C and finally ground and sieved in a 100 mesh. The obtained clay (bentonite exchanged with sodium) was denoted Na-Bent and was characterized using X-ray fluorescence (XRF) with a Magix Pro Philips PW2440 instrument. The CEC of Na-Bent was determined using the micro-Kjeldahl method [23].

Modification of the bentonite was carried out via the exchange of the Na^+ cations present in the clay (Na-Bent) with quaternary ammonium cations of the benzyltriethylammonium ion ($BTEA^+$). For the synthesis of the organoclay, two concentrations of the quaternary ammonium salt were used, 1.0 and 2.1 equivalents of the CEC of the Na-Bent. Sodium bentonite in water (2%, w/v) was mixed with a BTEAC solution until a clay-solution ratio of 1:100 g/mL was obtained. The suspensions were stirred at 250 rpm and heated to 45 °C for 10 h, and the solid was then separated by centrifugation, washed and dried at 35 °C for 12 h. The two organoclays are designated BTEA-1.0-Bent and BTEA-2.1-Bent, respectively.

Both Na-Bent and the two organoclays were characterized using X-ray diffraction, Fourier transform infrared spectroscopy (FT-IR) and N_2 adsorption–desorption at 77 K.

XRD patterns were obtained using a Rigaku Miniflex II diffractometer operating in Bragg–Brentano geometry using CuKα radiation (λ = 1.54056 Å) with 2θ steps of 0.02° at 4 s/step. The Fourier transformed infrared spectra (FT-IR) was recorded in the 4000–400 cm^{-1} range using a Perkin Elmer Spectrum BX spectrometer. FT-IR samples were prepared using the KBr pellet technique.

Nitrogen adsorption–desorption isotherms were obtained using a Micromeritics ASAP 2020 instrument at 77 K after outgassing the samples for 1 h at 30 °C followed by 8 h at 40 °C in vacuum. The specific surface area was determined using the Brunauer–Emmett–Teller method (BET), in which the total pore volume is derived from the amount of vapor adsorbed at the highest relative pressure closest to unity, assuming that the pores are filled with the liquid adsorbate [24,25], and the median pore width was determined using the Horvath-Kawazoe model assuming a slit pore geometry [26].

2.3. Immobilization of lipases on organoclay

C. rugosa lipase was immobilized via adsorption through a combination of hydrophobic, Van der Waals and electrostatic forces between the lipase and organoclay [18]. The enzyme solution was prepared in a phosphate buffer at pH 7 with a low ionic strength (100 mM); it was then heated for 2 h under gentle magnetic stirring, after which the support was added in a ratio of 10% (w/v); and this mixture was maintained under constant stirring and heating for 22 h. The samples were heated to 30 and 35 °C for BTEA-1.0-Bent and BTEA-2.1-Bent, respectively. Finally, the solids were washed and separated by centrifuging at 500 rpm. The support with the immobilized enzyme was dried for 12 h and then stored at 4 °C prior to use. A control experiment was carried out with the immobilized lipase in the unmodified bentonite, following the same procedure as employed with the organoclays.

2.4. Protein immobilization yield

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:

$$\text{Protein immobilization yield}(\%) = \left[\frac{C_i - C_f}{C_i}\right] \times 100$$

where C_i is the initial protein concentration in the lipase solution, and C_f is the final protein concentration in solution after immobilization. The protein concentration was measured according to the Biuret method using bovine serum albumin (BSA) as the standard [27].

2.5. Hydrolytic activity of the lipase

The hydrolytic activity of the immobilized lipase was assayed using an olive oil emulsion as the substrate according to the methodology described in the literature [28] with slight modification. The substrate was prepared by mixing 30 mL of olive oil, 2.1 g of gum arabic, 30 mL of deionized water and 40.8 mL of 100 mM phosphate buffer (pH 7). The reaction mixture containing 9 mL of the emulsion and 0.1 g of immobilized lipase was incubated for 5 min at 37 °C. The reaction was quenched by adding 10 mL of commercial ethanol. The fatty acids formed in the reaction were titrated with a 0.025 M potassium hydroxide solution in the presence of phenolphthalein as an indicator. A blank emulsion was used as a standard for the calculation.

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