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Protic ionic liquid as additive on lipase immobilization using silica sol–gel

Ranyere Lucena de Souza[a], Emanuelle Lima Pache de Faria[a], Renan Tavares Figueiredo[a], Lisiane dos Santos Freitas[b], Miguel Iglesias[c], Silvana Mattedi[c], Gisella Maria Zanin[d], Onélia Aparecida Andreo dos Santos[d], João A.P. Coutinho[e], Álvaro Silva Lima[a], Cleide Mara Faria Soares[a,*]

[a] UNIT, Universidade Tiradentes, Instituto de Tecnologia e Pesquisa-ITP, Av. Murilo Dantas, 300, Prédio do ITP, Farolândia, Aracaju, SE, Brazil
[b] UFS, Universidade Federal de Sergipe, Departamento de Engenharia Química, Avenida Marechal Rondon, São Cristovão, SE, Brazil
[c] UFBA – Universidade Ferderal da Bahia, Departamento de Engenharia Química, Rua Aristides Novis 2, Federação, Salvador, BA, Brazil
[d] UEM, Universidade Estadual de Maringá, Av. Colombo, 5790-zona 7, Maringá, PR, Brazil
[e] CICECO, Departamento de Química, Universidade de Aveiro, 3810-193 Aveiro, Portugal

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ABSTRACT

Ionic liquids (ILs) have evolved as a new type of non-aqueous solvents for biocatalysis, mainly due to their unique and tunable physical properties. A number of recent review papers have described a variety of enzymatic reactions conducted in IL solutions, on the other hand, to improve the enzyme's activity and stability in ILs; major methods being explored include the enzyme immobilization (on solid support, sol–gel, etc.), protic ionic liquids used as an additive process. The immobilization of the lipase from *Burkholderia cepacia* by the sol–gel technique using protic ionic liquids (PIL) as additives to protect against inactivation of the lipase due to release of alcohol and shrinkage of the gel during the sol–gel process was investigated in this study. The influence of various factors such as the length of the alkyl chain of protic ionic liquids (monoethanolamine-based) and a concentration range between 0.5 and 3.0% (w/v) were evaluated. The resulting hydrophobic matrices and immobilized lipases were characterised with regard to specific surface area, adsorption–desorption isotherms, pore volume (V_p) and size (d_p) according to nitrogen adsorption and scanning electron microscopy (SEM), physico-chemical properties (thermogravimetric – TG, differential scanning calorimetry – DSC and Fourier transform infrared spectroscopy – FTIR) and the potential for ethyl ester and emulsifier production. The total activity yields (Y_a) for matrices of immobilized lipase employing protic ionic liquids as additives always resulted in higher values compared with the sample absent the protic ionic liquids, which represents 35-fold increase in recovery of enzymatic activity using the more hydrophobic protic ionic liquids. Compared with arrays of the immobilized biocatalyst without additive, in general, the immobilized biocatalyst in the presence of protic ionic liquids showed increased values of surface area (143–245 $m^2\ g^{-1}$) and pore size (19–38 Å). Immobilization with protic ionic liquids also favoured reduced mass loss according to TG curves (always less than 42.9%) when compared to the immobilized matrix without protic ionic liquids (45.1%), except for the sample containing 3.0% protic ionic liquids (46.5%), verified by thermogravimetric analysis. Ionic liquids containing a more hydrophobic alkyl group in the cationic moiety were beneficial for recovery of the activity of the immobilized lipase. The physico-chemical characterization confirmed the presence of the enzyme and its immobilized derivatives obtained in this study by identifying the presence of amino groups, and profiling enthalpy changes of mass loss.

1. Introduction

Lipase (EC 3.1.1.3) is an important enzyme with a wide variety of applications in the food, fine chemical and pharmaceutical industries due to the multiplicity of reactions it catalyses, such as esterification, transesterification and hydrolysis [1–5]. Among them, the lipase from *Burkholderia cepacia* is distinguished by its ability to carry out organic synthesis, a feature of great interest to industry [6]. The economy of biocatalytic processes can be improved by improving the reuse and/or the enzyme itself, may improve performance of the enzyme under optimal conditions of reaction process (e.g., temperatures of alkalinity, acidity, organic solvents, and high), a requirement that has often retarded enzyme application in industrial chemical synthesis [7,8]. Therefore, numerous efforts have focused on the preparation of lipases

* Corresponding author. Tel.: +55 79 91322592; fax: +55 7932182190.
E-mail addresses: cleide.soares@pq.cnpq.br, cleide18@yahoo.com.br (C.M.F. Soares).

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in immobilized forms, involving a variety of both support materials and immobilization methods [6,9].

Compared to chemical methods to immobilize enzymes, physico-chemical methods, especially sol–gel encapsulation, making it a powerful tool for improving the properties of biomolecules immobilized for use as biosensors or biocatalysts [10,11]. A very large number of biomolecules have been immobilized by sol–gel techniques, and generally give better enzymatic activity and stability; however, there are also some disadvantages in the process of sol–gel immobilization [12]. One is shrinkage of the gel during the process of condensation and drying, which can cause denaturation of the enzyme. In addition, the slow diffusion of molecules of substrate for the enzyme within the sol–gel matrix hinders the catalytic activity of the immobilized enzyme in materials with a pore diameter smaller than 20 Å [13].

One way to overcome these drawbacks could be the use of additives to stabilize enzymes and assist in gel formation within arrays. Recently the use of aprotic ionic liquids as additives in the immobilization process has been reported, which could increase the activity and stability of immobilized enzymes by altering the hydration shell of the enzyme and reducing shrinkage of the gel [14]. They can also affect the physical properties of the gel by participating in condensation reactions with free silanol groups [14].

Studies of various additives such as ionic liquids (ILs) have recently been suggested as agents that can stabilize enzymes, protecting the hydration shell around the enzyme and/or causing conformational changes leading to permanent activation of the enzyme [15]. Lee et al. [16] reported that the process of immobilizing the *Candida rugosa* lipase using the sol–gel technique showed high stability and increased enzyme activity, about 10 times over that of the lipase in free form. The positive influence of the use of aprotic ionic liquids was also reported by Zarcula et al. [17] using $[C_8mim][BF_4]$ in the process of immobilizing the lipase from *Pseudomonas fluorescens* in hybrid sol–gel matrices as an additive; the results obtained with the immobilized biocatalyst showed total income generally exceeding 100%. The use of aprotic ionic liquids is also reported as additive on the activity and regioselectivity of *Rhizomucor miehei* lipase immobilized, showing that the affinity of the enzyme for the substrate increased in the presence of ILs more hydrophobic [18]. In fact, aprotic ionic liquids based on cations primarily in imidazolium and pyridinium showed satisfactory results in the immobilization of enzymes; however, the use of ionic liquids still has high costs of synthesis, which hinders the industrial application and can be toxic to enzyme [17,19].

Álvarez et al. [20] report that the ionic liquids obtained from amines, organic and inorganic acids, the so-called protic ionic liquids (PIL), feature low cost and simplicity of synthesis, favouring different applications including industrial applications. Moreover, the protic ionic liquids (PILs) have been demonstrated to be biocompatible with lipases, showing high activity and enantioselectivity in these media [21].

Potential applications have been identified for the use of PIL with proteins, such as dissolving hydrophobic ligands (e.g., ferrocene), to incorporate them into a protein crystal, improving the solubility of some proteins and improving monodispersity of proteins, as a precipitating agent and as an additive [22]. In particular, the potential of ionic liquids leads to implementing them as additives in the process of sol–gel immobilization, for the purpose of protecting against inactivation of enzymes due to release alcohol and shrinking of the gel during the encapsulation process.

This study aimed to immobilize the *B. cepacia* lipase in hydrophobic matrices obtained by a sol–gel technique in the presence of various protic ionic liquid alkyl chains with different C_2, C_3, C_4 and C_5, in addition to varying the content of a particular ionic liquid additive – C_5 (0.5–3.0%, w/v), which has hitherto not been reported, with physico-chemical properties of ammonium-based, assessing the total yield of recovered activity, potential production of ethyl esters and emulsifiers, and physical–chemical properties of the immobilized derivatives.

2. Experimental

2.1. Materials and reagents

Lipase from *B. cepacia* (Amano Lipase) was purchased from SIGMA – ALDRICH (Japan). The silane precursor tetraethoxysilane (TEOS) was supplied by Across Organic (NJ, USA) and used without further purification. Ethanol (minimum 99% pure), ammonia (minimum 28% pure), hydrochloric acid (minimum 36% pure) and gum Arabic were obtained from Synth (São Paulo, Brazil). Water was purified by reverse osmosis and deionized through a Milli-Q four-cartridge organic-free water purification system. The protic ionic liquids used are shown in Table 1. Other chemicals were of analytical grade and used as received.

2.2. Encapsulation of lipase from B. cepacia in sol–gel matrices

A methodology previously established by Patent PI0306829-3 [23] was used, and is briefly described as follows: 30 mL of TEOS was dissolved in 36 mL of absolute ethanol under an inert nitrogen atmosphere. To this, 0.22 mL of hydrochloric acid was dissolved in 5 mL of slowly added ultra-pure water, and the mixture was agitated (200 rpm) for 90 min at 35 °C. Lipase from *B. cepacia* (870.71 U) was dissolved in a solution of 10 ml of water, to which were simultaneously and separately added 1% (w/v) of the protic ionic liquids C_2, C_3, C_4 and C_5. As PIL-C_5 has proven to be the best additive (see Section 3.1), we also evaluated the effect of varying the amount of the additive added (0.5–3.0% w/v). 1.0 mL of ammonium hydroxide dissolved in 6.0 mL of ethanol (hydrolysis solution) were added to the sol–gel reaction, and the mixture was kept under static conditions for 24 h to complete polycondensation. The bulk gel was then washed with heptane and acetone and dried under vacuum at room temperature for 72 h. For comparison purposes, the encapsulated lipase (EN) from *B. cepacia* was prepared similarly in the absence of IL (EN-AIL), and pure silica sol–gel (PS) was prepared in the absence of the enzyme or additives. The sol–gel matrix immobilized lipases were designated: EN-C_2, EN-C_3, EN-C_4, EN-C_5 and EN-C_5-0.5, EN-C_5-1.0, EN-C_5-2.0, EN-C_5-3.0.

2.3. Enzymatic activity

Enzymatic activities of the free and immobilized lipase samples were assayed by the olive oil emulsion method according to a modification used by Soares et al. [24]. The substrate was prepared by mixing 50 mL of olive oil with 50 mL of gum Arabic solution (7% w/v). The reaction mixture containing 5 mL of the oil emulsion, 4 mL of sodium phosphate buffer (0.1 M, pH 7.0) and either free (1.0 mL, 0.1 mg mL^{-1}) or immobilized (≈250 mg) lipase was incubated in a thermostated batch reactor for either 5 min (free lipase) or 10 min (immobilized lipase) at 37 °C. A blank titration was done using a sample in which the enzyme was replaced with distilled water. The reaction was stopped by the addition of 2 mL of acetone–ethanol–water solution (1:1:1). The liberated fatty acids were titrated with potassium hydroxide solution (0.04 M) in the presence of phenolphthalein as an indicator. All reactions were carried out in triplicate. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of free fatty acid per min (μmol min^{-1}) under the assay conditions (37 °C, pH 7.0, 80 rpm).

Analyses of hydrolytic activities were carried out on the lipase loading solution and bioencapsulated preparations to determine the total activity recovery yield, Y_a (%), according to Eq. (1):

$$Y_a = \frac{U_s}{U_o} \times 100 \quad (1)$$

where U_s is the total enzyme activity recovered on the support and U_o is the enzyme units offered for immobilization.

2.4. Ethyl esters and emulsifier production

Transesterification reactions catalysed by the lipase from *B. cepacia* were performed under the conditions proposed by Noureddini et al. [10]. The transesterification reactions were carried out in batch reactors, submerged in a thermostatic bath to keep each mixture at constant temperature and under agitation. The reaction was initiated by mixing soybean oil and ethyl alcohol (ratio 1:15.2), 0.075 g water, and lastly the free or immobilized *B. cepacia* (EN-AIL or EN-C_5), as PIL-C_5 proved to be the most potent additive (see Section 3.1). The equivalent of 38.3 U of enzyme were added to each sample. Aliquots were removed at different time intervals and analysed by a GC gas chromatograph equipped with a CARBOVAX (30 m × 0.25 mm × 0.25 μm) column. Column temperature was initially kept at 140 °C for 1 min, increased to 180 °C at a rate of 4 °C per minute, maintained for 2 min, then increased to 230 °C at a rate of 10 °C per minute, where it was maintained for a further 10 min. The temperatures of the injector and detector were set to 250 °C. Ethyl ester conversion (%) was defined as the observed amount of product divided by the theoretical yield if all the soybean oil was converted.