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Organofunctionalized silica gel as a support for lipase

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ABSTRACT

This work described the preparation of silica gel modified with cyanuric chloride. The modified silica gel was tested for the ability to immobilize *Burkholderia cepacia* lipase. Contact times ranging from 4 to 24 h were investigated to determine the optimal lipase immobilization time. Following immobilization, the enzyme activity was assessed by the hydrolysis of *p*-nitrophenylpalmitate (*p*NPP). Elemental analysis data revealed that 0.4 mmol of cyanuric chloride were anchored per gram of support, demonstrating the successful incorporation of the triazine molecule onto the silica surface. The tests of reusability and storage indicated that the enzyme-modified silica-gel (immobilization at 24 h) was more stable under reaction conditions than the other systems. The activity assays indicated high rates of enzymatic formation of *p*-nitrophenol (*p*-NP), demonstrating a maximum activity retention of 87%.

1. Introduction

The use of lipases (triacylglycerol acylhydrolases) as biocatalysts in organic reactions is of great interest in the processing of oils and fats because these enzymes exhibit the ability to catalyze the hydrolysis of esters, liberating fatty acids and glycerol, as well the reverse reaction, that is, ester synthesis [1–3]. Recently, these enzymes have been investigated in biocatalysis, particularly in the synthesis of pharmaceuticals, to obtain pure enantiomer compounds and to produce biodiesel by transesterification [1,3–5].

The ability of lipases to catalyze a variety of reactions is a result of their regioselectivity, enantioselectivity, and chemoselectivity as well as with their stability in organic solvents [4–6]. The use of lipolytic enzymes in bioprocesses is particularly advantageous when the enzymes are immobilized onto a solid support, enabling the facile recovery and reuse of the immobilized enzyme over several cycles of the reaction without a significant loss of biological activity. In addition, immobilized lipases may show superior activity compared to the free enzyme, including greater thermal and pH stability [7–9].

Lipases can be immobilized on a variety of supports, including materials that are organic (cellulose, chitin, chitosan), inorganic (silica, clay, alumina), hydrophilic or hydrophobic, and by several methods including ion adsorption, covalent binding, crosslinking, encapsulation, or immobilization in membranes [1,10–12]. Both the support and the immobilization method are critical in determining the thermal stability and the mechanical and chemical properties of the resulting biocatalyst. The cost of immobilization must also be considered [13]. Accordingly, inorganic supports exhibit numerous advantages compared with organic supports, due to their superior mechanical and thermal properties [14–16]. In terms of the immobilization method, covalent bonding provides a more effective interaction between the enzyme and the support, leading to a more chemically and thermally stable enzyme interaction and minimizing desorption of the enzyme during the biocatalytic process [17–19].

Due to the diverse conditions of biocatalysis reactions, it is important to consider a variety of solid supports, immobilization methods and reaction parameters to maximize the stability of the enzyme and to minimize losses in catalytic activity.

Previously, both natural and synthetic media have been used as matrices for lipase immobilization to obtain a biocatalyst with high catalytic efficiency that can be recovered and reused for several reaction cycles.

In this study, the synthesis and characterization of silica gel modified with cyanuric chloride was investigated. This silica gel was used to immobilize a commercial lipase from *Burkholderia cepacia*. The catalytic behavior of the immobilized enzyme was evaluated by the hydrolysis of *p*-nitrophenylpalmitate (*p*-NPP).

2. Materials and methods

2.1. Materials

The following materials were used: chromatographic silica gel (Sigma-Aldrich) with a 70–230 mesh particle size, a median diameter of 60 Å, and a pore volume of 0.75 $m^3\ g^{-1}$; (3-aminopropyl) trimethoxysilane 97% (Aldrich); cyanuric chloride 99% (Acros

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Organics); Amano Lipase PS from *B. cepacia* (Sigma-Aldrich); bovine serum albumin BSA (Sigma); protein Biuret reagent (Ambresco®); and Folin–Ciocalteau's phenol reagent (Sigma-Aldrich). The chemicals 1,4-dioxane, ethanol 99%, and isopropanol 98% were used as solvents and were obtained from Vetec. Toluene was purchased from Synth. The substrates *p*-nitrophenylpalmitate (Sigma-Aldrich) and gum arabic (Aldrich) were used for the activity assays.

2.2. Functionalization of the silica gel support

The silica gel was functionalized using an organosilane containing an amino functional group as a modifying agent. Prior to the chemical modification of the inorganic matrix, the silica was activated by heating at 150 °C under vacuum for 12 h. This procedure was necessary to remove water from the silica surface, allowing the silanol groups to react with the silylating agent. Once activated, the silica (Sil-At) reacted with 3-aminopropyltrimethoxysilane (15.0 mL, 86.0 mmol) in a reflux of toluene (150.0 mL) under mechanical agitation and a nitrogen atmosphere at 120 °C for 72 h. The resulting aminopropylsilica (Sil-N) was washed sequentially with toluene, ethanol, and deionized water.

2.3. Reaction of cyanuric chloride with aminopropylsilica

The organosilane-modified silica gel (Sil-N) was allowed to react with cyanuric chloride (7.5 g, 40.7 mmol) under mechanical stirring in 150.0 mL of a mixture comprising 1,4-dioxane/toluene in a 4:1 ratio at 12–20 °C for 12 h. This synthesis was based on previous procedure [20]. The material was then filtered and washed with toluene and acetone. The resulting functionalized solid was termed Sil-NCC.

2.4. Characterization of modified silicas

The functionalized silicas were characterized by the elemental analysis of CHN and Cl (Fisions Instruments Elemental Analyzer, Model EA — 1110 CHNS-O). Infrared spectroscopy (Nicolet 380 Spectrophotometer Thermo) was performed using KBr pellets and a spectral range of 4000 to 400 cm^{-1}. Thermogravimetry (DTG-60H Shimadzu Thermal balance) measurements were collected under a nitrogen atmosphere with a flow rate of 50 mL min^{-1} and a heating rate of 10 °C min^{-1}.

2.5. Immobilization of lipase on silica modified with cyanuric chloride

The immobilization of lipase from *B. cepacia* was performed at varying time intervals (4, 8, 12, 16, 20, and 24 h) to investigate the influence of contact time on the amount of immobilized protein and the enzyme activity. A 10.0 mL volume of enzyme solution (2.00 mg mL^{-1}) dissolved in phosphate buffer (66.0 mmol L^{-1}, pH 7.2) was added to 150 mg of the functionalized support. The system was allowed to react under stirring at 150 rpm and 25 °C. Following immobilization, the solids were washed thrice with 5.0 mL of immobilization buffer to remove any weakly adsorbed enzyme residues.

2.6. Determination of lipase loading

The amount of protein present in the supernatant solutions (following immobilization) and the solutions leached with the enzyme (following washing of the support) was spectrophotometrically determined by the method of Lowry (1951) [21] using bovine serum albumin (BSA) as a standard. The samples containing the enzyme were measured by absorbance at 660 nm using a UV/VIS spectrophotometer (TECNAL Spectrophotometer 2000 UV/VIS) against a pH 7.2 blank buffer solution without enzymes. The amount of protein bound to the support was calculated as the difference between the amount of protein dissolved in the solution prior to immobilization and the amount of protein remaining in the filtered solutions and the buffer wash following immobilization. The mass of protein retained per gram of support was calculated using Eq. (1).

$$p = \frac{C_i V_i - (C_s V_s - C_l V_l)}{m_s} \quad (1)$$

where p represents the amount of bound protein (mg g^{-1} support), C_i is the initial concentration of the protein (mg mL^{-1}), C_s is the concentration of the protein (mg mL^{-1}) present in the filtered solutions, C_l (mg mL^{-1}) is the concentration of the leached protein, V_i (mL) is the volume of the solution used to immobilize the enzyme, V_s (mL) is the volume of the supernatant solution, V_l is the volume (mL) of the support washing solution, and m_s is the support mass. All experiments were performed in triplicate, and an average was calculated from the experimental data.

2.7. Enzyme activity

The enzyme activities of free and immobilized lipase were spectrophotometrically determined using the method reported by Winkler and Stuckmann [22]. The procedure involved the hydrolysis of *p*-nitrophenylpalmitate in the presence of free (2.00 mg mL^{-1}) or immobilized enzyme (30.0 mg) by incubation in a pre-heated medium (37 °C, pH 8.0) for 15 min. The reaction releases the yellow compound *p*-nitrophenol (*p*NP), which exhibits an absorption peak at 410 nm. Samples were measured against a blank containing a pH 8.0 buffer without enzymes. The retention of enzyme activity was calculated according to Eq. 2, using the molar extinction coefficient $\varepsilon = 15\ kcm^2\ mol^{-1}$. A unit of enzyme activity was defined as the amount of the enzyme needed to produce 1.0 μmol min^{-1} of *p*NP under the conditions of the test. All experiments were performed in triplicate, and an average was calculated from the experimental data.

$$\%U_r = \frac{U_{exp} \times 100}{U_i}. \quad (2)$$

Here, U_r is the recovered activity, U_{exp} is the experimentally observed activity from the immobilized enzyme, and U_i is the theoretical maximum activity.

2.8. Reusability

Reusability was evaluated in terms of the recovery of activity over the course of *p*NPP hydrolysis experiments. For this study, we used the derived enzyme, which exhibited superior immobilization efficiency and retention of enzymatic activity. The immobilized biocatalysts were subjected to five consecutive cycles under the same reaction conditions used to assess the hydrolytic activity of the substrate. Following each reaction cycle, the samples were washed consecutively with isopropyl alcohol and pH 7.2 phosphate buffer.

2.9. Storage stability

The lifetime of the immobilized biocatalysts was evaluated by determining the residual enzyme activity under the reaction conditions established in Section 2.7 after 30 days of storage at 4 °C without the use of solvent. Following the storage period, the enzymes were employed in the hydrolysis of *p*-NPP for five consecutive batches. After each reaction interval, the support was washed thrice with 5.0 mL of pH 7.2 phosphate buffer. The residual activity was determined as described in Section 2.7.

2.10. Statistical treatment

To minimize the propagation of errors, all glass instruments used in the determinations were calibrated. For each step of the procedure,