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Bio-inspired silica lipase nanobiocatalysts for the synthesis of fatty acid methyl esters

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ABSTRACT

Green production of biodiesel *via* enzymatic transesterification necessitates technological improvements for practical implementation. Heterogenization of biocatalysts has long demonstrated its advantages in biocatalytic processes. In this work, a lipase from *Thermomyces lanuginosus* (TL) has been immobilized on biomimetic silica nanoparticles using two different strategies: *in situ* entrapment and adsorption/covalent surface immobilization. Variables such as mass of nanobiocatalyst, amount of immobilized enzyme, and activity per gram of silica were studied following a factorial design for the *in situ* immobilization. The influence of the enzymatic load on the stability and activity of the catalyst was studied for both the surface immobilized and the entrapped lipase. Immobilized preparations were characterized and assayed in the production of fatty acid methyl esters (FAMES).

The entrapped nanobiocatalysts were more stable and active than the soluble TL, the commercial immobilized TL and the surface immobilized counterparts. However, surface immobilized lipase reached a maximum yield of 88% in the synthesis of FAMES from canola oil and methanol, surmounting from the yield obtained with the commercial immobilized TL by 10%. Accumulated specific productivity for the entrapped biocatalyst reached 65.6 μmoles FAME/g catalyst/min after repeated batch operation. Biomimetic silica demonstrated its versatility and robustness as a support for TL immobilization in the synthesis of FAMES.

1. Introduction

Lipase catalyzed biodiesel synthesis has several advantages over alkaline catalysis. The mild reaction conditions of temperature and pH and high specificity and selectivity towards transesterification substrates allow a greener transformation and ease the downstream processing of products [1]. However, the cost of lipases and its relatively low reaction rates prevent them from competing at industrial level with alkaline catalysis. In a time where environmental consciousness is starting to change the scene of the chemical industry at the pace of decisions from regulatory agencies, it seems pertinent to continue searching for cost effective alternatives for enzymatic conversions.

Enzyme heterogenization through immobilization is one of the main strategies to counterbalance the cost of a biocatalyst. It provides increased activity and stability, facilitates the recovery and reuse of the biocatalysts and allows alternative process configurations [2,3]. In order to consolidate the use of biocatalysts as a feasible alternative for the industrial production of biodiesel, it is necessary to design cost effective strategies that increase their stability and activity, and reduce the cost of the immobilization support.

The use of silica as a support for enzyme immobilization has the advantage of being more environmentally friendly than acrylic resins and chemically more resistant to organic solvents and fouling [4–7]. It also allows the synthesis of nanometric supports with increased superficial area leading to catalysts with less mass transfer limitations [8,9]. This is particularly important considering that one of the major disadvantages of immobilized lipases for biodiesel synthesis is the longer reaction times required than in alkaline catalysis.

The silica synthesized by diatoms is an interesting starting point for enzyme immobilization since it occurs under mild conditions compatible with biological activity [8,10]. Biomimetic or bio inspired silica is the *in vitro* silica formation through reactions derived or similar to those occurring *in vivo*. It produces nanostructured particles and is carried out at close to neutral pH and room temperature under aqueous conditions. Addition of enzymes in the synthetic mixture has allowed the *in situ* entrapment of multiple enzymes [11,12]. The fungal *Thermomyces lanuginosus* lipase (TL) has been recently the focus of attention of Novozymes that has already in the market products based on this enzyme [13,14]. TL is a monomer of 39 KDa with a wide pH activity profile that, as many other enzyme of its class, has its hydrophobic active site

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covered by a "lid". The enzyme suffers a structural rearrangement when exposed to hydrophobic substrates rendering the active site accessible for substrate binding and leaving the enzyme in an open configuration. This phenomenon is the basis for the so called interfacial activation of lipases which activity increases dramatically upon binding to the lipid surface formed by their water-insoluble substrates.

Although the biochemistry of the TL lipase has been extensively explored, studies on the immobilization of this specific lipase compared with others widely applied in industry (*i.e.* the lipase from *Candida antartica*) have been scarce which opens up an opportunity for academia to contribute to its technological use.

In this work, we have evaluated the immobilization of the lipase from *T. lanuginosus* in biomimetic silica supports through two different strategies, comparing its hydrolytic activity, stability under simulated reaction conditions and behavior in the synthesis of biodiesel.

2. Materials and methods

2.1. Materials

Lipase from *Thermomyces lanuginosus* (lipase TL), p-nitrophenyl propionate (pNPP), polyethyleneimine (MW 25,000) and 3 Å beads molecular sieves (4–8 mesh) were purchased from Sigma Chemical Co. (St. Louis, USA). Methanol (Merck, Darmstadt, Germany) and other solvent used were of analytical grade. 100% canola oil was of food grade (Mazola- Watts, Santiago, Chile). Protein concentration of the soluble enzyme was determined by the bicinchoninic acid assay using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA). Lipozyme® TL was from Novozyme (Bagsværd, Denmark).

2.2. Hydrolytic enzyme activity assays

This assay was performed by continuously measuring the increase in the absorbance at 348 nm produced by the released *p*-nitrophenol in the hydrolysis of 0.4 mM pNPP in 25 mM sodium phosphate buffer at pH 7.0 and 30 °C.

One international unit of hydrolytic activity on pNPP (IU_H) was defined as the amount of enzyme necessary to hydrolyze 1 μmol of pNPP per minute at the above described conditions.

Activity of lipase TL was 1090 ± 83 IU/mL, corresponding to a specific activity of 53 IU/mg protein.

2.3. Biocatalyst drying

Drying of biocatalysts was performed by speed vacuum at 35 °C with a SpeedVac SPD 121 P. Entrapped biocatalyst were dried for 60 min with 100% of the activity recovered after the process. Biocatalyst immobilized on heterofunctional nanoparticles were dried for 10 min and activity recovered was 30%.

2.4. GC analysis

The methyl ester contents in the reaction mixture were quantified using a GC7890. A gas chromatograph (Agilent Corp., California USA) was connected to a Supelco OMEGA –WAX column (30 m x 0.25 mm x 0.25 m) and a flame ionizing detector (FID). The injector and detector temperatures were adjusted to 250 °C and 270 °C, respectively. Nitrogen was used as the carrier gas. The column temperature was maintained at 170 °C for 2 min, and then increased to 240 °C at the rate of 2.5 °C/min, and maintained at 240 °C for 20 min. Percent biodiesel yield was defined as the amount of fatty acid methyl esters produced divided by the initial amount of canola oil.

2.5. Lipase immobilization by in situ entrapment in biomimetic silica

0.5 mL of polyethyleneimine (PEI) and 0.5 mL of tetramethylortosilicate (TMOS) were added to 1 mL of enzymatic solution in sodium phosphate buffer at pH 8.0. The mixture was left under agitation 10 min and centrifuged for 5 min at 12,000 rpm. The nanoparticulated pellet was washed with 0.5 M NaCl in 5 mM sodium phosphate pH 7.0.

The influence of the reactants on the synthesis was studied using a complete factorial design of three variables and two levels. The selected variables to study were the conentration of TMOS, PEI and sodium phosphate. The levels included high (+) and low (-) concentrations of the reactants. Concentrations were 904 mM and 197 mM for TMOS, 10.8 mM and 2.2 mM for PEI and 50 mM and 2.5 mM for sodium phosphate.The standard protocol involved high levels of the three reactants. Protein concentration in the assay was 4.5 mg/mL. The effect of these variables was evaluated in terms of weight of catalyst, protein immobilization yield and activity immobilization yield.

Immobilization yield in terms of protein (Y_P) and activity (Y_A) were calculated according to Eqs. (1) and (2), respectively:

$$Y_P(\%) = \frac{P}{P_0} \times 100 \quad (1)$$

$$Y_A(\%) = \frac{A}{A_0} \times 100 \quad (2)$$

where Po represents the amount of offered protein, P the amount of loaded protein in the biocatalyst, Ao the offered enzyme activity and A the activity expressed in the biocatalyst.

2.6. Synthesis of heterofunctional biomimetic silica

2.6.1. Synthesis of biomimetic silica nanoparticles

TMOS was hydrolyzed with 1 mM HCl in 154:1000 (v:v) ratio. 0.25 mL of freshly hydrolyzed TMOS was added to 0.25 mL of a 10% PEI solution in 100 mM sodium phosphate buffer pH 8.0. The particles formed were centrifuged and washed three times with 25 mM sodium phosphate buffer pH 7.0.

2.6.2. Biomimetic silica nanoparticle functionalization

1 g of dried silica support was incubated with 30 mL of 5% octyl trimethoxysilane (OTMS) and 5% (3-Glycidoxypropyl)methyldiethoxysilane in toluene under reflux at 105 °C for 5 h. The incubation was followed by a wash with acetone and resuspension in 30 mL of 0.1 M sulphuric acid under reflux at 95 °C for 2 h. After a wash with distilled water, oxidation was carried out with 30 mL of 0.1 M $NaIO_4$ for 2 h at 25 °C in order to generate aldehyde groups. The supernatant was used to calculate the concentration of aldehyde by $NaIO_4$ consumption.

The obtained nanoparticles were washed, filtered and dried at 60 °C for 16 h [15].

2.7. Lipase immobilization on heterofunctional biomimetic silica

An enzymatic solution was prepared in 25 mM sodium phosphate buffer pH 7.0 with different protein concentrations and incubated with the support in a 40:1 vol to g of support ratio.

The suspension was gently stirred at 25 °C and samples were periodically withdrawn until constant protein content and hydrolytic activity in the supernatant. The mixture was centrifuged for 5 min at 12,000 rpm and the particles were suspended in 100 mM sodium bicarbonate buffer pH 10.0 in a 1:40 w/v ratio and incubated for 30 min at 25 °C under gentle stirring. The supernatant was withdrawn after centrifugation and the solids were suspended (1:40 w/v ratio) in a 1 mg/mL solution of $NaBH_4$ in 100 mM sodium bicarbonate buffer pH 10. The mixture was incubated during 30 min under stirring at 4 °C. The suspension was centrifuged for 5 min at 12,000 rpm. The particles were washed twice by resuspension (1:40 w/v ratio) in 25 mM sodium phosphate buffer pH 7.0. For each immobilization, Y_P and Y_A were determined.