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Immobilization of *Candida rugosa* lipase by adsorption onto biosafe meso/macroporous silica and zirconia

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ABSTRACT

Lipase from *Candida rugosa* was immobilized by adsorption onto laboratory prepared supports, silica SBA-15 and zirconia. The adsorption process was studied as a function of pH in terms of percent of adsorbed lipase, enzyme activity and zeta potential of support and enzyme. Several analytical approaches such as laser Doppler electrophoresis, Fourier transform infrared spectroscopy (FTIR) and field emission scanning electron microscopy (FESEM) showed that the lipase was successfully immobilized onto both supports. The ζ-potential data suggest that the adsorption efficiency does not depends on the sign but on the magnitude of the surface charge of adsorption partners, and therefore underline the importance of their dispersion stability. Adsorption to material surface altered enzyme characteristics. ν_{max} for the lipase immobilized onto silica and zirconia were 4.8-fold and 3.6-fold lower than that of the free lipase, respectively. The K_m showed no alteration of enzyme-substrate affinity on zirconia support, whereas the enzyme immobilized on silica had 3.6 times lower affinity. Thermostability at 60 °C of the lipase was improved 12-fold on zirconia and 4-fold on silica. Finally, in examining reusability, the immobilized lipase retained more than 90% of initial activity after eight reuses on both supports.

1. Introduction

A challenge facing the chemical, pharmaceutical and allied industries in the 21st century is the transition to green processes in order to avoid or minimize the generation of waste and the use of toxic and/or hazardous materials [1]. Enzymes have many benefits to offer in this respect. The great potential of enzymes as bio-derived catalysts has been based on their relative ease of production from renewable resources, their selectivity and specificity, mild reaction conditions (ambient temperature and pressure, physiological pH) and low energy consumption. To overcome structural instability and allow repeated use, enzymes have been successfully immobilized on different supports using various techniques which offer easier separation and recyclability in the substrate transformation, thereby making production more economically effective [2].

Immobilization methods can be divided into three categories: cross-linking, entrapment and binding to a support [3]. Each method can influence the enzyme and its properties: activity, stability and selectivity [4]. Cross linking offers high stability but it can cause steric hindrance by the substrate [5], while enzyme immobilization by entrapment in microspheres or membranes is often followed by inefficient contact between enzyme and substrate, and the exit of products [6,7]. Finally, the method based on physical adsorption of enzyme onto the surface of water-insoluble carriers [8] causes little or no conformational change of the enzyme or destruction of its active site. This method is usually simple and inexpensive if a suitable carrier is used. As a result of the weak binding force between the enzyme and the carrier, this method, however, has a disadvantage, in that the adsorbed enzyme leaks from the carrier during the repeated use. Considering that the underlying reason for enzyme immobilization, besides improvement in stability, is its recyclability [3], it seems that the adsorption has been somewhat neglected in comparison to other methods.

Lipases (EC 3.1.1.3) are widely employed to catalyze hydrolysis, alcoholysis, esterification and transesterification of carboxylic esters. They catalyze reactions with high specificity, regio- and enantioselectivity, and therefore, are used in a wide range of

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applications, both in industry and research. Lipases of microbial origin are the most widely used group for practical applications. They are among the most promising and important biocatalysts for carrying out reactions in both aqueous and non-aqueous media [9–11].

Different inorganic supports have been used for the immobilization of lipases. *Candida antarctica* lipase was immobilized onto a ceramic membrane for enhancing both hydrolytic and synthetic reactions [12]. The use of magnetic nanoparticles improved stability and facilitated separation of *Candida rugosa* (*C. rugosa*) lipase, and increased thermal stability of *Pseudomonas cepacia* lipase for the production of biodiesel [13,14]. Furthermore, lipase was immobilized in hierarchically ordered mesocellular mesoporous silica by enzyme adsorption, followed by glutaraldehyde crosslinking with an enhanced stability [15]. Also, silica and silica-coated magnetic nanoparticles were effective for lipase immobilization [16].

Excellent activity and reusability of the alkaline lipase from *Pseudomonas fluorescens*, immobilized by adsorption on multi-wall carbon nanotubes has been reported recently [17].

C. rugosa lipase is an enzyme commercialized by several suppliers (Sigma, Roche, Amano) and widely used in a variety of biotechnological applications, such as production of carbohydrate esters of fatty acids, stereoselective synthesis of pharmaceuticals and different applications in food and flavor production, biosensor production and biodiesel production [18,19].

Silica and zirconia together with alumina and titania are among the main ceramic surfaces used in biomaterials [20]. Porous silica has been extensively used for the immobilization of enzymes aiming at their use as biocatalysts [21]. Several advantages, such as high surface area, tailored pore size and volume, and high stability together with non-toxic and biocompatible properties of mesoporous silicas [22–24], as well as the excellent mechanical and chemical properties of zirconia (in comparison with traditional support materials such as alumina and silica) [25,26], have made them prevalent biomaterials in contemporary biotechnology.

Silica and zirconia powders have been used for immobilization of *C. rugosa* by covalent bonding [27,28], or by adsorption on previously modified, hydrophobilized, supports [29].

Successful immobilization by adsorption of an enzyme strongly depends on surface characteristics of both enzyme and support material. To develop an efficient immobilized enzyme, several steps are required according to the guide published by Hudson et al. [30]. Briefly, both materials, enzyme and matrix, should be characterized in detail, immobilization probed and stable preparation of the immobilized enzyme onto a support characterized relative to free enzyme.

Following this approach in the current study, two inorganic materials, laboratory synthesized ceramic powders, were examined as supports for the immobilization of *C. rugosa* lipase by adsorption. This investigation reports the first successful attempt of immobilization of *C. rugosa* lipase by simple adsorption onto mesoporous silica SBA-15 and macroporous zirconia powders without any modification either of the lipase or the supports. Optimization of the adsorption process was performed. Zeta potential, FESEM and FTIR analyses were used to determine surface charge, morphology and chemical properties of the immobilized lipase. The effects of the immobilization on the lipase characteristics were also investigated, confirming that the primary reason for immobilization – improvement in the enzyme stability and recyclability – was achieved.

2. Materials and methods

2.1. Materials

Lipase from *C. rugosa* (lyophilized powder, Type VII, nominal activity 746 U mg^{-1}) and *p*-nitriphenyl palmitate (*p*-NPP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

The powders chosen as supports for this study were mesoporous SiO_2 (silica) SBA-15 and macroporous ZrO_2 (zirconia), both laboratory prepared, according to the published protocols [31,32]. SBA-15 was synthesized by a templating method. Pluronic123 acid (non-ionic triblock copolymer) was used as a structure directing agent and tetraethoxysilane (TEOS) as the silica source. Zirconia was prepared by plasma spray jet synthesis. The zirconia precursor was firstly atomized into droplets then injected into a high temperature plasma flame, and powder particles were collected on an electrostatic precipitator.

2.2. Methods for materials characterization

In silico analysis of the lipase structure was performed using the database UniProtKB at Expasy Proteomics Server, where lipase from *C. rugosa* was found as entry LIP1_CANRU (P20261). The protein was analyzed using ProtParam tool [33]. The VEGA ZZ program was used for display, analysis and calculation of the open structure of *C. rugosa* lipase, PDB entry 1crl [34]. The polar surface area (PSA) was calculated taking into consideration both polar and non-polar atom surfaces. Non-polar atoms are C and H bonded to C, while polar atoms are O, S, N, P and H not bonded to C. As a part of the Vega ZZ package, Probka was used for protein pKa predictions [35].

Measurements of the zeta (ζ) potential were performed using a Zetasizer Nano ZS (Malvern, UK). The instrument software calculates the ζ-potential from the electrophoretic mobility of the particles measured by laser Doppler electrophoresis. The ζ-potential was determined for free and immobilized lipase as well as for the supports in 10 mM buffers (acetic, phosphate and Tris) in the pH range 5–9 at 25 °C.

Fourier transform infrared spectroscopy (FTIR) was used to examine the support before and after the immobilization of lipase. The spectra were recorded using FTIR Nicolet 6700 (Thermo Scientific, USA) in transmission mode between 400 and 4000 cm^{-1}. The platinum-ATR (Attenuated Total Reflectance) sampling module with diamond crystal was used.

Field emission scanning electron microscopy (FESEM), using a Tescan Mira3 XMU (Czech Republic) at 20 kV and Brunauer, Emmett and Teller (BET) analysis were used to study the support morphology before and after adsorption of lipase, and support porosity. Prior to FESEM analysis, the samples were coated with Au-Pd alloy using a spatter coater. To determine the specific surface area and porosity of zirconia, adsorption and desorption of nitrogen were measured on the ZrO_2 sample, using the gravimetric Mc Brian method, as reported for silica [31].

2.3. Lipase immobilization

The effect of pH on the adsorption of lipase was investigated (in the pH range 5–9) using the following 10 mM buffer solutions: acetate buffer (pH 5.0), phosphate buffer (pH 6.0, 6.4, 7.0, 7.6) and Tris buffer (pH 7.0, 7.6, 8.0, 8.6, 9.0).

Silica and zirconia were prepared for immobilization as follows: suspensions containing 4 mg support ml^{-1} in selected buffer were de-agglomerated for 10 min by sonication. Lipase solution (2 mg/ml in selected buffer), was added to de-agglomerated support, the resultant suspension was sonicated for 10 min and immobilization was carried at room temperature under mild stirring (90 rpm). After 60 min, the immobilized lipase was precipitated by centrifugation at 10,000 rpm for 10 min using a microcentrifuge (Denver Instruments, USA). The immobilized enzyme was washed twice to remove the excess of unbound enzyme, dried for 1 h at room temperature and used for the assay.

Kinetics of the adsorption was studied for 180 min using 10 mM Tris buffer pH 7.6 and the same lipase/support ratio (w/w) 1/2.