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Strategies of covalent immobilization of a recombinant *Candida antarctica* lipase B on pore-expanded SBA-15 and its application in the kinetic resolution of (*R*,*S*)-Phenylethyl acetate

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ABSTRACT

A recombinant *Candida antarctica* lipase B expressed in *Pichia pastoris* (LIPB) was immobilized on pore-expanded SBA-15 previously modified 3-amino-propyltriethoxysilane (APTES) and activated with two bifunctional reagents, glutaraldehyde (GA) or divinylsulfone (DVS), producing the biocatalysts: SBA-15-APTES-GA-LIPB and SBA-15-APTES-DVS-LIPB, respectively. After LIPB immobilization, both preparations were then modified with glutaraldehyde, producing the biocatalysts: SBA-15-APTES-GA-LIPB-GA, SBA-15-APTES-DVS-LIPB-DVS. Alternatively, LIPB was immobilized on SBA-15-APTES-DVS at pH 10.2 and the biocatalyst was named SBA-15-APTES-DVS-LIPB-pH10. The different biocatalysts were assayed to check the effect of the immobilization strategies on the stability and in the substrate specificity during the kinetic resolution of (R,S)-Phenylethyl acetate. The thermal stability of some new preparations were higher than LIPB adsorbed on SBA-15 (SBA-15-LIPB) and LIPB immobilized on Glyoxyl-agarose. High conversions in the enzymatic kinetic resolution were obtained (43–50%) for all biocatalysts studied. Regarding activity and stability, the SBA-15-APTES-DVS-LIPB-pH10 was the most successful strategy, since, in first cycle, the maximum conversion was obtained (50%), and the biocatalyst remained active and enantioselective even after five successive cycles.

1. Introduction

As a result of the interest in green processes, the enzyme market grew considerably in the last years, reaching USD 4 billion in 2012 and a 8.3% annual growth is expected until 2018 [1]. Although they are specific, which reduces the formation of undesired products when compared to traditional chemical synthesis [2], they present some drawbacks to be used as industrial biocatalysts [3]. To overcome this drawbacks, enzyme immobilization have been widely used in order to increase stability of the enzyme [4], to allow the reuse of the biocatalyst, the application in continuous process [5] and among others [6].

Lipase B from *Candida antarctica* is extensively used in organic synthesis [7,8], because it has high stability in organic solvents and high thermal stability, allowing the catalysis to occur at high temperatures [9]. In addition, the lipase B from *Candida antarctica* is an effective biocatalyst for the kinect resolution of racemic compounds, producing enantiomerically pures bioproducts, due to its high stereoselectivity [10–12]. In this work, a recombinant *Candida antarctica* lipase B, expressed in *Pichia pastoris* using PGK as a constitutive promoter [12,13], was immobilized. LIPB is a site-specific glycosylated protein at Asn 74 [14] and the *N*-glycosylation works

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as a sugar tag on the protein and can be used for oriented immobilization [14,15]. Furthermore, glycosylated proteins have some advantages in protein chemistry, influencing their correct folding, biological activity and tertiary structure stability [15]. Although this lipase has many advantages, its use in industrial process is still reduced due to the low stability of the soluble form of the enzyme.

Lipase immobilization have been often reported in the literature [8,16,17] as a toll to stabilize proteins, and covalent immobilization has been described [18–22]. In this technique, the enzyme-support interactions are very strong and the leaching of the enzyme is minimized, producing more stable biocatalysts [23]. Many reactive groups may be used for the covalent immobilization of enzymes, among these, the glutaraldehyde reactive group is a powerful crosslinker, a widely used molecule to produce CLEAs [24]. Glutaraldehyde-activated supports are normally very versatile, because of the presence of hidrophobic groups, ionic groups and covalent moieties for the enzyme to interact [25–27]. However, in amino preactivated supports, at long incubation times, the enzyme-support covalent attachment is more probable to be achieved [25]. On the other hand, divinylsulfone-activated supports were recently proposed as an alternative to the epoxy-supports, because the enzyme groups envolved in the immobilization are similar [27]. Divinylsulfone reactive groups, in specific conditions, are able to immobilize the enzyme strongly by multipoint covalent attachment [28].

Many materials have arised as a support for immobilization and new protocols of immobilization have been used [27,28]. Mesoporous silica materials are extensively used in catalysis, gas adsorption and drug delivery processes [30–32]. SBA-15 is a highly ordered mesoporous silica which has been widely used as a support in enzyme immobilization [3], either by adsorption [33] or covalent binding [22]. In addition, enzyme immobilized onto mesoporous silica can be used in fixed bed reactors, but, for large scale industrial applications, large size particles are required in order to reduce the flow resistance [34]. For small scale applications, microchannel reactors are also used [35], which have many advantagens than conventional reactors, such as: large surface area to volume ration and rapid mass and heat transfer [3].

In this work, a pore-expanded mesoporous silica (SBA-15) was chosen for LIPB immobilization due to some environmental and technical requirements, namely: high surface area, large pore volume [36], inert structure, non-toxicity and high biocompatibility [22,37]. Moreover, the SBA-15 has parallel pores and benefits arising from the combination of micro and mesoporosity [36,38]. In this work, the pore-expanded SBA-15 was synthesized with ammonium fluoride, and with this modification, this material becomes more suitable for enzyme immobilization purposes as it enhances the mass transfer [39] by the formation of shorter channels [40].

In this context, the aim of this paper was to evaluate the immobilization a recombinant *Candida antarctica* lipase B on the pore-expanded SBA-15 by covalent attachment. For the best of our knowledge, it is the first time that divinylsulfone was used to activated SBA-15. Last but not least, the biocatalysts catalyzed the kinetic resolution of (*R,S*)- Phenylethyl acetate to produce the secundary alcohol, (*R*)-1-phenylethanol. This compound is a high value aggregate product, since it is important intermediate for valuable medicines and fine chemicals [41,42]. This process, the use of divinylsulfone as activating agent for the production of many robust biocatalysts supported on SBA-15, is described for first time and innovative results were obtained, specially regarding activity and stability. Furthermore, the use of those biocatalysts for the kinetic resolution of (R,S)-Phenylethyl acetate expanded the library of biocatalysts for this given application.

2. Materials and methods

2.1. Materials

The homemade *Candida antarctica* lipase B expressed in *Pichia pastoris* (LIPB, 0.77 mg of protein per mL) was provided by the Research Group of the Engineering of Biological Systems from Department Biochemistry Engineering at Federal University of Rio de Janeiro, Brazil [12,13]. Agarose 6 BCL, glutaraldehyde solution 25%, divinylsulfone, *p*-nitrophenyl butyrate (*p*-NPB) and 3-aminopropyltriethoxysilane 98% (APTES) were purchased from Sigma Chemical Co (St. Louis, MO, USA). All reagents and solvents were obtained in analytical grade.

2.2. Methods

2.2.1. Preparation and modification of SBA-15 for enzyme immobilization

In this work, SBA-15 was investigated as support for the covalent immobilization of LIPB. The SBA, previously impregnated with APTS, was activated by different strategies using glutaraldehyde (GA) and divinylsulfone (DVS).

2.2.1.1. Synthesis of SBA-15. Pore-expanded SBA-15 was prepared by a hydrothermal route according to Santos et al. [36], with some modifications. In a typical experiment, 4.6 g of Pluronic P123 ($PEO_{20}PPO_{70}PEO_{20}$) were dissolved in 160 mL HCl solution (1.3 M), followed by the addition of 0.052 g of NH_4F under magnetic stirring (25 °C). After the reagents dissolution, 9.76 g of tetraethoxysilane (TEOS) were added. The mixture was stirred for 24 h and transferred to an autoclave for subsequent reaction at 100 °C for 48 h. Then, the solid was filtered, dried for 24 h and calcined at 550 °C, at a heating rate of 3 °C.min^{-1}, for 5 h in order to remove the director agent of the structure (P123).

2.2.1.2. Preparation of APTES-impregnated SBA-15. In this work, the impregnation of 3-(Aminopropyl) triethoxysilane (APTES) on the surface of SBA-15, an amine modification, was performed by post-synthesis method. In the procedure, 3 mL APTES per gram of support were added, in the presence of 40 mL of toluene and 20 mL of absolute ethanol, by sonication for 3 times during 5 min at room temperature. Subsequently, the material was washed with methanol and ethanol to remove the excess of APTES. The resultant material was named SBA-15-APTES.

2.2.1.3. Activation with glutaraldehyde (GA). SBA-15-APTES was activated with glutaraldehyde according to Barbosa et al. [25], with some modifications. In this procedure, 1 g of SBA-15-APTES was suspended in 10 mL of phosphate buffer (200 mM and pH = 7), containing 15% (v/v) of glutaraldehyde. The mixture was kept under agitation for 15 h at 25 °C. Finally, supports were washed 5 times with phosphate buffer (5 mM and pH 7) to remove the excess of glutaraldehyde. The support was named SBA-15-APTES-GA.

2.2.1.4. Activation with divinylsulfone (DVS). Activation of SBA-15-APTES was conducted by suspending 1 g of SBA-15-APTES in 10 mL of buffer solution (200 mM) containing divinylsulfone [29]. The experiments were performed for 35 min at 25 °C. Different conditions of activation were studied: concentration of DVS (5, 10, 15, 20 and 25% w/v) and the pH of buffer solution (7, 10, 12.5). Afterwards, the support was excessively washed with distilled water and stored at 4° C. The support was named SBA-15-APTES-DVS.

2.2.1.5. Characterization of SBA-15. SBA-15 was characterized by a combination of several techniques. X-ray powder diffraction patterns (XRD) were recorded on a PANalytical model EMPYREAN