Preparation of Micro-porous Silica Xerogel with Magnetic Properties and its Application to Penicillin G Acylase Immobilization

S.M.S.A. Bernardino[1], P. Fernandes and L.P. Fonseca

Institute for Biotechnology and Bioengineering, CEBQ, IST, Lisboa, Portugal
[1] susana1972@gmail.com, luis.fonseca@ ist.utl.pt

Introduction

The bioconversion processes have been widely studied and according to the progresses on biotechnology they can replace chemical synthetic processes in the field of pharmaceutical, food, and chemistry specially in the production of high valuable conpounds. Use of bioconversion processes utilizing immobilized enzyme to produce antibiotics are most active (Kim et al., 2006).

Semi-synthetic penicillins (e.g. amoxicillin, ampicillin, ticarcillin) and cephalosporins (e.g. cephalexin, cefaclor, cefadroxil) correspond to 65% of the ever rising worldwide production of antibiotics, exceeding 45 000 tons in 2000. Penicillin G Acylase (PGA) from *E. coli* is currently used to catalyze the synthesis of β-lactam antibiotics and hydrolysis of penicillin G (Giordano et al., 2006).

The enzymatic synthesis of these antibiotics on a commercial basis is only feasible if immobilized biocatalysts are used, given the cost of enzyme production. Immobilization, however, often brings along diffusion limitations, which have a negative effect on the overall reaction rate. Such drawback has been reported as most significant in the enzymatic synthesis of β-lactam antibiotics (Kallenberg et al., 2005).

The materials used for biocatalyst immobilization are required to be chemically and mechanically stable, non-toxic, biologically inert and often hydrophilic, features that are typical of silica supports. Common immobilization methods using silica supports require preliminary support activation for the ensuing successful ionic or covalent attachment of the enzyme. The modifications performed often lead, however, to loss of catalytic activity (O´Neil et al., 2002).

The sol-gel process, which allows the room temperature synthesis of silica glasses, has been employed extensively for the entrapment of both organic and inorganic molecules. A well-established sol-gel processing technique consists in hydrolyzing the adequate precursors in aqueous solutions to produce soluble hydroxylated monomers, followed by polymerization and phase separation to produce a hydrated metal or semi-metal oxide hydrogel. Removal of water from the wet gel, which is usually accompanied by changes in the structure of the pores and of the gel network, results in a porous xerogel. The most widely used precursors are alkyl-alkoxysilanes (Reetz et al., 1998; O´Neil et al., 2002). Entrapment of lipases in a silica matrix has proved to be effective and efficient. When this

approach was applied to penicillin G acylase, however, the activity recovery was rather poor (10%), which the authors ascribed to diffusion limitation in the silica matrix (Kallenberg et al., 2005).

The main goal of this work is the immobilization of PGA in a sol-gel silica matrix with magnetic properties. A mechanically stable carrier, based on porous xerogels silica matrixes starting from tetramethoxysilane (TMOS) was produced. Diffusion limitations are minimized given the diameter (25 nm- 50 μm) of the micro-carriers obtained. Immobilization of PGA in these micro-carriers allowed for an immobilization yield of 95-100%, whereas the recovered activity was 45-70% at 37 ºC, as determined by the pH STAT method. These values of activity were kept constant during at least six months.

Material and methods

PGA solution (26.6 mg dm^{-3}, 35 U mg^{-1}) from *Escherichia coli*, tetramethoxysilane (TMOS), sodium dioctyl sulfosuccinate (AOT), magnetite nanopower, were all purchased from Sigma-Aldrich. Isooctane was supplied from Riedel de Haën. Penicillin G was obtained from Fersinca Gb. All other reagents used were either laboratory or analytical grade.

Enzyme assay and Operational stability

One unit of PGA activity (U) for the soluble and immobilized enzymes is defined as the amount of enzyme required to produce 1 μmol of 6-APA (6-Amino Penicillanic Acid) per minute. Enzyme activity was determined in a small batch stirred reactor with automatic pH correction by the pH STAT method (Fonseca et al., 1993), using a 4% (w/v) penicillin solution in 20 mmol dm^{-3} pH 8.0 phosphate buffer at 37ºC. To evaluate the operational stability of the biocatalyst, Penicillin G hydrolysis was performed during 10 minutes at 37 ºC and pH 8.0 (pH STAT method), using the immobilized PGA in sol-gel in aqueous medium.

Assay for protein concentration

The concentration of protein in the enzyme solution before immobilization and in the supernatant and washings, after immobilization, was determined through mass quantification related to a calibration curve of PGA absorbance at 280 nm. The amount of protein linked to the support was calculated by mass balance.

A*ctivity retention*

The percentage of activity retention was calculated as the ratio between the specific activities of the enzyme immobilized on the support and of the free enzyme solution.

Sol-gel

A solution containing 100 μL TMOS (2.32 mol dm^{-3}) and 40 μL HCl (1.37 mmol dm^{-3}) was sonicated in a Transsonic T 460 sonicating water bath for 10 min until the hydrolysis reaction was complete (sol-

solution). In a typical immobilization procedure, 75 µL of PGA was suspended in 85 µL of magnetic suspension -10% (w/v) in 100 mmol dm^{-3} phosphate buffer, pH 7.5 - and the whole mixed with the sol-solution. To obtain micro-particles and minimize diffusion limitations, 300 µL of the sol-gel solution with enzyme was immediately added to 6 mL of 150 mmol dm^{-3} AOT/isooctane solution, before gelation. The resulting mixture was placed under vortex for 2 min, washed twice with 100 mmol dm^{-3} phosphate buffer, pH 7.5 and aged at 2-8 ºC during one week. The micro-particles obtained were suspended in 1 mL of the same phosphate buffer. The particles are either used or stored at 2 – 8 °C.

SEM

Dry particles of biocatalyst were put on a carbon tape and analyzed in a Field Emission Scanning Electron Microscope (Jeol JSM-7001F).

Results and Discussion

In this work, particular emphasis was given to advances in penicillin G acylase immobilization by entrapment in a silica matrix with magnetic properties. Mechanically stable xerogel carriers containing magnetite were produced from tetramethoxysilane (TMOS), to yield an immobilized biocatalyst in the form of nano- and micro-carriers, with sizes roughly ranging from 25 nm to 30 µm, as determined by SEM. A typical SEM micrograph of micro-carriers is given in figure 1. The presence of magnetite considerably eased the recovery of the biocatalyst, from the reaction media, through the use of a magnetic concentrer (figure 2).

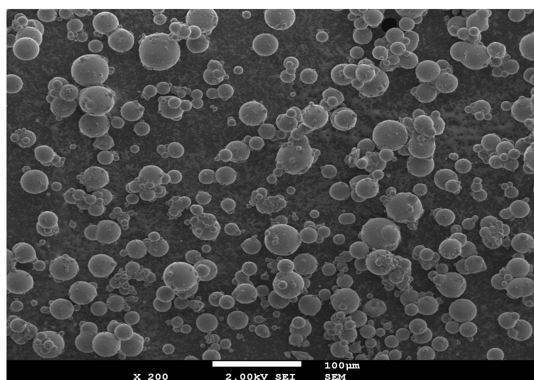

Figure 1: SEM micrograph of the sol-gel matrices with encapsulated magnetite and PGA (bar match 100 µm).

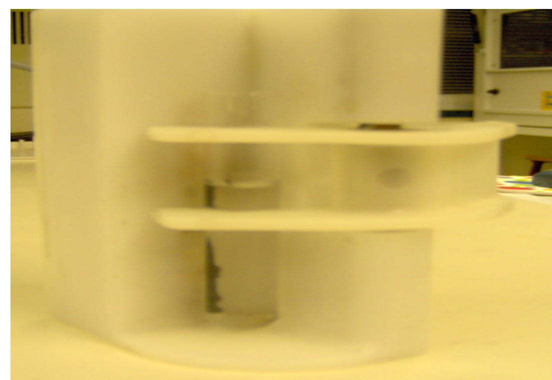

Figure 2: Magnetic particles recovery on a magnetic concentrer.

Immobilization of PGA in these carriers allowed for an immobilization yield of 95-100%, whereas the recovered activity was 45-70% at 37 ºC, as determined by the pH STAT method, depending on the aging, which clearly exceeds results (10%) reported in a previous work on PGA immobilization in sol-gel (Kallenberg et al., 2005). Temperature and pH dependency are similar to free and immobilized PGA (see Figure 3 and 4). The energy of activation also remained roughly unchanged following

immobilization, for 10.5 and 10.8 kcal mol^{-1}, for free and immobilized enzyme, respectively, were determined from Arrhenius plots. Immobilized PGA retained 65% activity throughout 10 consecutive runs for penicillin G hydrolysis suggesting adequate biocatalyst stability.

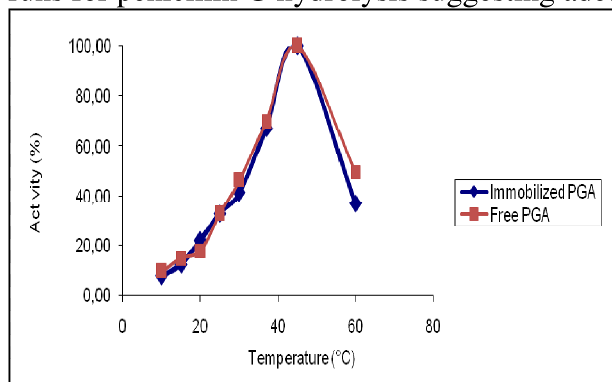

Figure 3: Relative activity versus temperature at pH 8,0 (pH STAT method) for free and immobilized enzyme.

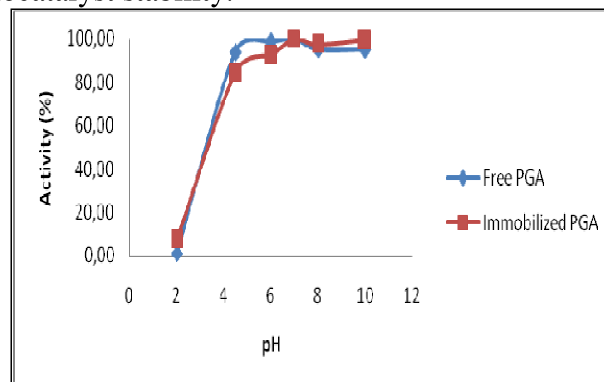

Figure 4: Relative activity versus pH at 37 ºC (pH STAT method) for free and immobilized enzyme.

Conclusions

Enzyme immobilization was carried out using penicillin G acylase from *Escherichia coli* in a silica xerogel matrix with magnetic properties. The enzyme is catalytically active and can carry out hydrolytic reactions efficiently. Novel developments such as additive effects are being tested to prevent the observed biocatalyst deactivation under prolonged exposure to operational conditions, aiming to develop a biocatalyst that can be recycled many-fold, a mandatory feature to render enzymatic catalysis economically feasible.

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